# AFLATOXIN ELIMINATION WORKSHOP

St. Louis, Missouri

October 24-25, 1994



Draft

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Aflatoxin is recognized as a serious food safety hazard by most countries of the world. Producing food free of aflatoxin today requires a truly national effort and, particularly, the cooperation of both government and industry. The Agricultural Research Service (ARS) and the commodity groups representing peanuts, corn, cottonseed, and tree nuts recognize the importance of a strong national research effort to eliminate aflatoxin as a food safety threat.

This Aflatoxin Elimination Workshop, held in St. Louis, Missouri, is the seventh such yearly meeting held to review the ARS supported aflatoxin research and provide a forum for interested scientists to come together to discuss common problems and their potential solutions among themselves and with members of the industry. Thus, although many of these scientists are performing very fundamental studies researchers and representatives of industries affected by aflatoxin who attend the workshop gain a very clear idea of where their research is leading and the impact it will have on society. Also, this workshop provides the opportunity for gains in cost effectiveness of research by the recognition of common approaches and by sharing relevant information across commodities. This workshop has come to be recognized as the premier national meeting for advances leading to methods to eliminate aflatoxin.

Research advances were reported in every session of the Workshop. Molecular biology continues to provide basic information that can be used by each prevention strategy to ensure its success. With the characterization of the regulatory gene for aflatoxin biosynthesis, we now have the tools for detecting how aflatoxin biosynthesis is turned on and what parameters, environmental or otherwise, are required for onset of this process. Highly competitive natural strains, even though they are aflatoxin nonproducers, have many of the enzymes and genes for biosynthesis while noncompetitive strains do not produce these enzymes. This suggests a role of some aflatoxin-related genes in survival of the fungus.

Sources of resistance for incorporation in breeding programs have been identified in both peanut and corn breeding lines. Complete freedom from fungal infection of almond seed continues to be observed in genotypes with a completely sealed endocarp suggesting this as the most promising resistance breeding strategy. A transformation protocol has been developed that allows the routine introduction of foreign genes into peanut tissue and the regeneration of transgenic plants. A number of groups have transformed or will in the near future transform walnut, peanut, and cotton with genes that encode lytic peptides/proteins, the genes that are currently most promising to confer resistance to *A. flavus*. Analyses of both infecting fungal strains and aflatoxin content of infected cottonseed demonstrated that the extent of contamination of cottonseed both in test plots and in the untreated commercial crop was related to both dispersal and the complexity of infection. Reduction in toxin content in coinfected seed is probably attributable to competition for both infection sites and nutrients.

Studies demonstrated that sap beetles, which are a known vector of *Aspergillus* species to several crops, will also carry biocontrol organism to corn. An expert system, which is well along in development to prevent aflatoxin contamination of peanut, is now being developed to minimize contamination of corn grown in the South.

Most of the research is performed by the ARS, however an important addition to this core effort is provided through a competitive award program provided by Congressional appropriations. This program is a unique effort of the ARS and representatives of the peanut, corn, cotton, and tree nut industries. By extending the opportunity for the best university scientists to join the highly focused multithrust program, the rate of progress toward the elimination of aflatoxin is enhanced.

Jane F. Robens

JANE F. ROBENS
Agricultural Research Service
Workshop Program Coordinator

November 22, 1994

**TABLE OF CONTENTS**

**AFLATOXIN ELIMINATION WORKSHOP**
**ABSTRACTS OF PRESENTATIONS**

## AFLATOXIN ELIMINATION WORKSHOPS

| | |
|---|---|
| New Orleans, LA | 1988 |
| Peoria, IL | 1989 |
| St. Louis, MO | 1990 |
| Atlanta, GA | 1991 |
| Fresno, CA | 1992 |
| Little Rock, AR | 1993 |
| St. Louis, MO | 1994 |
| Atlanta, GA | 1995 |

## COOPERATING COMMODITY GROUPS

| | |
|---|---|
| PEANUTS: | National Peanut Council |
| CORN: | National Corn Growers Association<br>American Corn Millers Federation<br>Corn Refiners Association |
| COTTONSEED: | National Cottonseed Products Association<br>National Cotton Council |
| TREE NUTS: | Prune, Raisin & Walnut Marketing Board<br>Almond Board of California<br>California Pistachio Commission<br>DFA of California |

# CROP RESISTANCE - CONVENTIONAL BREEDING

Identification of Peanut Germplasm with Resistance to Preharvest Aflatoxin Contamination. C. C. HOLBROOK[1], D. M. Wilson[2] and M. E. Matheron[3]. [1] USDA-ARS, Coastal Plain Exp. Sta., Tifton, GA; [2] Dept. of Plant Path., Univ. of GA, Tifton, GA; [3] Dept. of Plant Path., Univ. of AZ, Sommerton, AZ.

Preharvest aflatoxin contamination (PAC) is one of the most serious challenges facing the U.S. peanut industry. The development of peanut cultivars with resistance to PAC would be a valuable tool in helping to alleviate the problem. The objective of this research was to examine peanut germplasm for resistance to PAC. Over 90% of the accessions in a core collection were examined for resistance in preliminary field screening trials using five replications, and 75 were selected for further examination. Additional studies were conducted to examine germplasm with potential for having resistance to PAC. These studies were grown in 1993 in a RCB with ten replications at Yuma, AZ and Tifton, GA. The plots were inoculated with a mixture of A. flavus and A. parasiticus about 60 days after planting and subjected to drought stress for the 40 days immediately preceding harvest. One study consisted of 19 lines with resistance to other fungal pathogens. One of these lines had significantly lower PAC than florunner. Another study consisted of 18 lines with drought tolerance. Six of these lines exhibited at least a 90% reduction in PAC in comparison to florunner. Previous research has indicated that the linoleic acid content of a substrate can affect aflatoxin production by Aspergillus. Recently, peanut breeding lines with reduced linoleic acid content have been developed. The level of aflatoxin contamination in seven breeding lines with reduced linoleic acid content (less than 5% of total fatty acid composition) was compared to the check cultivar, Florunner, in a separate field study. Aflatoxin contamination levels were greater in Yuma than in Tifton, however, the genotypes*location interaction was not significant. Averaged over locations, Florunner exhibited a PAC level of 3022 ppb. All of the seven breeding lines with low linoleic acid exhibited a PAC level of less than 50% the level observed in Florunner. The breeding lines F1344 (45ppb), F1315 (50ppb) and F1316 (137ppb) had significantly ($p \leq 0.05$) lower PAC levels than Florunner.

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Aflatoxin Accumulation in Seed of Ten Peanut Genotypes Inoculated with Aspergillus flavus and A. parasiticus.

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Ten peanut genotypes were evaluated for A. flavus/A. parasiticus sporulation and aflatoxin accumulation after incubation at 25C in the laboratory. Twenty-five seed per replication of each genotype were brought to 25% moisture content and inoculated with a mixture of A. flavus NRRL 5520 and A. parasiticus NRRL 2999. Five replications were used for six collection dates (0, 2, 3, 4, 5 and 10 days). After four days seed of all genotypes contained sporulating colonies of A. flavus/A. parasiticus. There were significant differences between number of seed with sporulation among genotypes. Multiple regression of aflatoxin accumulation over time helped demonstrate that there were significant differences among genotypes as well as the linear and quadratic relationships. Entry PI 290626 accumulated the least amount of aflatoxin and exhibited a linear relationship for the $\ln(B_1+1)$ and $\ln(G_1+1)$ multiple regression. All other genotypes exhibited quadratic relationships. Using regression of $B_1$ and $\ln(B_1+1)$ and $\ln(G_1+1)$ data the entries with the least aflatoxin accumulation were PI 290626, and the low linoleic acid genotypes F1348 and F1316. These data are preliminary at this stage; however, it appears that we may have a model system to reliably assess resistance to aflatoxin accumulation in potentially resistant peanut genotypes.

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# AFLATOXIN CONTAMINATION OF 'RESISTANT' AND 'SUSCEPTIBLE' CORN BELT HYBRIDS GROWN AT WESLACO, TEXAS, 1993

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In 1993, six aflatoxin 'resistant' and two 'susceptible' hybrids in field trials (1990-92) at Bloomington, IL and Union City, TN were planted 'late' (April) at Weslaco, TX to assess their performance when grown in an environment conducive to aflatoxin formation (e.g. high evening temperatures > 75 C during interval of kernel filling). Ears in the late-milk to early-dough stage of kernel maturity were wound-inoculated with A. flavus at three sites on the ear using the punch-drill & pipecleaner method. At harvest, approximately twenty (20) undamaged kernels surrounding each wound-inoculation site were examined for BGYF and aflatoxin. Elimination of the individual wound-inoculated kernels from each sample removes a source of highly aflatoxin-contaminated damaged grain that has little relevance to the resistance of maturing grain to A. flavus infection and subsequent aflatoxin contamination. Contrasts were made with undamaged grain sampled from uninoculated rows (Table 1). Three of the 'resistant' hybrids having the same female parent [CIBA Seeds #00544 x 00565: #00554 x 00526; #00544 x 00558] were contaminated with substantially less aflatoxin than the 'susceptible' hybrids in both treatments. Three of the 'resistant' hybrids (CIBA Seeds #30488; #30671; #00630 x 00565) performed poorly at Weslaco, pointing to the importance of evaluating the performance of corn varieties in an aflatoxin conducive environment. Curiously, wound-inoculation did not produce higher levels of aflatoxin contamination in B73 x MO17, CIBA Seeds #30663, or #30488. This could suggest that all available sites suitable for aflatoxin production (i.e. germ region of individual kernels having a tear in seed coat, etc.) were colonized by fungal strains from the naturally occurring A. flavus population. This research was performed through a Cooperative Research and Development Agreement No. 58-3K95-M-67.

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Table 1. Aflatoxin (ppb) contamination of undamaged grain sampled from wound-inoculated and uninoculated rows, Weslaco, TX, 1993.

| | AA | V | D | I | S | J | BB | U |
|---|---|---|---|---|---|---|---|---|
| Wound-Inoculated Rows | 646 | 503 | 270 | 215 | 276 | 520 | 1506 | 893 |
| Uninoculated Rows | 640 | 72 | 47 | 45 | 152 | 130 | 1563 | 703 |

| CIBA Inventory # | CODE | |
|---|---|---|
| #30488 | AA | Demonstrated Resistance in Corn Belt |
| #30671 | V | " " " |
| 00554 x 00565 | D | " " " |
| 00544 x 00526 | I | " " " |
| 00554 x 00558 | S | " " " |
| 00630 x 00565 | J | " " " |
| B73 x Mo17 | BB | Highly Susceptible in Corn Belt |
| #30663 | U | " " " |

a. Harvest date August 9, 1993.
b. Average aflatoxin values (ppb) for kernel samples from three replicate rows of 20 corn plants.

# EVALUATION OF VARIOUS CORN HYBRIDS FOR RESISTANCE TO *ASPERGILLUS* EAR ROT AND AFLATOXIN

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In 1993, eight commercial hybrids (six classified as resistant - G4666, G4665, G4543, G4680, P3732, P3165, and two classified as susceptible - B73 x Mo17, and P3377) received from ICI Seeds, twenty-two hybrids (four commercial yellow dent hybrids and eighteen specialty corn hybrids, including white, high amylose, waxy, and blue corn hybrids) received from Pioneer International, and five experimental $F_1$ hybrids (including Mo17 x Tex6) developed at the University of Illinois, were evaluated for *A. flavus* ear rot, kernel infection, and aflatoxin. MP420 x Tx601 and GT-MASgk were included as resistant checks and MASpwnf was included as a susceptible check.

In 1994, the eight commercial hybrids, five experimental $F_1$ hybrids, and the resistant and susceptible checks were reevaluated for *A. flavus* ear rot and aflatoxin. Also, five specialty hybrids received from Cornuts Inc., Oakland, CA., ten commercial yellow dent hybrids received from Illinois Foundation Seeds Inc. (IFSI), Champaign, IL., and one experimental $F_1$ hybrid (Tex6 x B73) developed at the University of Illinois were evaluated for *A. flavus* ear rot and aflatoxin.

Plants were inoculated (1) 20 days after midsilk with inoculum prepared using a mixture of four isolates (NRRL 6536, 6539, 6540, and a 1988 isolate from Illinois) of *A. flavus*. Forty to fifty days after inoculation, ears were husked, and a visual rating of 1-10 (1=10%, 10=100% of the inoculated area rotted) was determined for each ear and averaged for each plot. Plots were harvested 60-70 days after midsilk, dried, machine shelled, and ground. Fifty randomly selected sub-samples of kernels were removed for kernel planting. Aflatoxin analyses were done on grain samples from the bulked corn grain using indirect competitive enzyme-linked immunosorbent assay.

In the 1993 experiment, mean ear rot ratings and aflatoxin values for the thirty-eight genotypes were 4.6 (range of 1.9-8.5) and 111 ng/g (range of 0-696 ng/g), respectively. In 1994, mean ear rot ratings and aflatoxin values for the thirty-two genotypes were 4.7 (range of 2.1-7.1) and 64 ng/g (range of 9-154 ng/g), respectively.

Of the six ICI hybrids classified as "resistant", only P3165 consistently had moderately low levels of aflatoxin. Two of the hybrids (G4543 and G4666) had ear rot and aflatoxin values comparable to the susceptible checks. G4666 had a moderately high level of aflatoxin in 1994. The Pioneer yellow dent hybrids had relatively low levels of ear rot, but had moderate to high levels of aflatoxin. The Pioneer yellow waxy hybrids had moderately low levels of ear rot and low to moderately low levels of aflatoxin. The Pioneer high amylose hybrids had moderately high levels of ear rot, but low levels of aflatoxin. The blue corn and white corn hybrids generally had the highest ear rot ratings and aflatoxin.

Aflatoxin levels were much lower in 1994 (i.e. many of the ICI commercial hybrids evaluated in 1994 had aflatoxin levels one-third to one-half of their respective levels in 1993), probably due to the cool environmental conditions. The IFSI hybrids had moderate to high levels of ear rot, but relatively low levels of aflatoxin. Classification of the IFSI hybrids was difficult due to the narrow range of aflatoxin values. Generally, the cornuts hybrids had both low levels of ear rot and aflatoxin. The experimental $F_1$ hybrids Mo17 x Tex6 and B73 x Tex6 had levels of resistance to *A. flavus* ear rot and aflatoxin that were comparable to the resistant checks Mp420 x Tx601 and GT-MASgk. Inbred Tex6 appears to be a promising source of resistance.

## Literature Cited

1. Campbell, K.W., and White, D.G. 1994. An inoculation device to evaluate maize for resistance to ear rot and aflatoxin production by *Aspergillus flavus*. Plant Dis. 78:778-781.

# Selection of Corn Genotypes Resistant to *Aspergillus flavus* Ear Rot, Kernel Infection, and Aflatoxin

K.W. Campbell and D.G. White, University of Illinois, Urbana, IL, G.E. Scott and Natale Zummo, USDA-ARS, Mississippi State, MS, and N. Widstrom, USDA-ARS, Tifton, GA

In 1991, 1,041 and 949 $F_1$ crosses of a diverse group of corn inbreds with the susceptible inbreds Mo17 and B73, respectively, were evaluated for resistance to *A. flavus* ear rot following inoculation in Urbana, IL. From the $F_1$ hybrid evaluation studies, 18 inbreds crossed with B73 and 17 inbreds crossed with Mo17 were selected for further study (2). In 1992 and 1993, the 35 $F_1$ crosses and the 32 parental inbreds were reevaluated for *Aspergillus* ear rot, percent kernel infection, and aflatoxin. In addition, 15 selected $F_1$ hybrids were evaluated in Mississippi and Georgia by G. E. Scott, N. Zummo, and N. Widstrom, respectively.

Inoculation technique and isolate(s) of *A. flavus* varied with location. In the Illinois experiments, plants were inoculated 20 days after midsilk and rated for visual ear rot 40-50 days after inoculation (1). One hundred kernels and 50 kernels were plated from each plot in 1992 and 1993, respectively. Aflatoxin analyses were done on grain samples from the bulked ears using an indirect competitive enzyme-linked immunosorbent assay. Results indicate that sources with high levels of resistance to *Aspergillus* ear rot and aflatoxin have been identified. Inbreds Tex6, LB31, and CI2 consistently had the highest levels of resistance to *A. flavus* infection (both ear rot and kernel infection) and aflatoxin. When included in experiments, Mp420 x Tx601 and Mas:gk also had high levels of resistance. Several inbreds (i.e. 75-R001 and T115) contributed high levels of resistance in crosses with B73 or Mo17, but were rated as susceptible as inbreds per se. This would indicate that these inbreds have genes for resistance not found in either B73 or Mo17.

Genotypes with low ear rot ratings generally had lower levels of aflatoxin contamination. Pearson correlation coefficients between ear rot rating and aflatoxin ranged between $r = 0.52$ and $r = 0.80$ in the inbred and $F_1$ hybrid experiments. Generally, Spearman rank correlation coefficients indicated a consistent relationship between ear rot rankings and aflatoxin rankings. In this study, kernel infection and aflatoxin were not significantly correlated, possibly due to the low numbers of kernels that were plated.

The inheritance of resistance is currently being studied and resistance is being incorporated into commercially acceptable corn inbreds for public release.

1. Campbell, K.W., and White, D.G. 1994. An inoculation device to evaluate maize for resistance to ear rot and aflatoxin production by *Aspergillus flavus*. Plant Dis. 78:778-781.

2. Campbell, K.W., and White, D.G., Toman, J., and Rocheford, T.R. 1993. Sources of resistance in $F_1$ corn hybrids to ear rot caused by *Aspegillus flavus*. Plant Dis. 77:1169.

# Inheritance of resistance to *Aspergillus flavus* and aflatoxin with sources of resistance identified at the University of Illinois

D.G. White, K.W. Campbell, and T.R. Rocheford, University of Illinois, Urbana, IL.

Genetic Resistance

In 1991, 18 and 17 inbreds crossed with the susceptible inbreds B73 and Mo17, respectively, were identified as potentially having high levels of resistance to *Aspergillus* ear rot (1). In 1992, inheritance of resistance to *Aspergillus* ear rot was studied in the 35 crosses utilizing the parental inbreds, $F_1$, $F_2$, and backcross to the susceptible parent generations. The $F_3$ generations were also evaluated for 10 of the crosses. In 1993, 11 of the crosses with the highest levels of resistance were reevaluated using the parental inbreds, $F_1$, $F_2$, $F_3$, and backcross to the susceptible and resistant generations. Five $F_2$ and three $F_3$ populations were also evaluated for aflatoxin. In 1994, additional studies were done with four of the resistant sources using parental inbreds, $F_1$, $F_2$, $F_3$, resistant and susceptible backcross generations, selected $F_4$ lines, and backcross to the susceptible self generation. In 1994, the number of $F_3$ families was greatly increased, and to study the inheritance of resistance to aflatoxin per se, all generations of two sources of resistance will be analyzed for aflatoxin.

Plants were inoculated 20 days after midsilk with inoculum prepared from four isolates of *A. flavus* (2). Approximately 40-50 days after inoculation, ears were husked and a visual rating of 1-10 (1=10%, 10=100% of the inoculated area rotted) was determined for each ear and averaged for each plot. Ears from plots were harvested 60-70 days after midsilk, dried, machine shelled, bulked and ground for aflatoxin analysis. Randomly selected subsamples of kernels were removed for kernel plating. Aflatoxin was quantified by an indirect competitive enzyme-linked immunosorbent assay. Significance of additive, dominance, and the three digenic epistasis effects were determined on unweighted means of ear rot ratings by generation mean analysis. Broad-sense heritability estimates and minimum number of effective factors were calculated.

Additive and dominance gene action were of primary importance in resistance to Aspergillus ear rot. Additive effects were of primary importance in the crosses B73 x LB31, B73 x CI2, and Mo17 x Tex6, whereas, genic dominance was of primary importance in B73 x L317, Mo17 x OH513, and B73 x 75-R001. Crosses of Mo17 x 75-R001 and Mo17 x H103 were nonsignificant in the additive dominance model. Aflatoxin frequency distributions of $F_3$ families of the Mo17 x Tex6 and B73 x LB31 populations, were highly skewed toward the resistant parent indicative of genic dominance. Both ear rot ratings of various generations and aflatoxin values of $F_2$ and $F_3$ generations indicate various levels of transgressive segregation in most crosses. Broad-sense heritabilities ranged from 12-68, and number of effective factors ranged from -1.16 to +7.72. Results from generation mean analyses, broad-sense heritabilities, effective factors, and Aspergillus ear rot and aflatoxin frequency distributions indicate that selection for resistance to Aspergillus ear rot and aflatoxin should be effective when appropriate sources of resistance are used. Results indicate that lines Tex6 and LB31 have high levels of resistance which are controlled by a relative low number of genes. Inbred 75-R001, even though susceptible as an inbred per se, has genes for resistance not found in either B73 or Mo17 and can be used to improve B73 and Mo17 type inbreds. Inbred CI2 has a high level of resistance and even though the resistant is not highly dominant it can be crossed into B73 type inbreds. Several other sources of resistance need to be studied in greater detail. Phenotypic mass selection should give fairly rapid progress in pedigree selection and backcrossed breeding for resistance.

## RFLP Analyses

Restriction fragment length polymorphism (RFLP) analyses were performed to identify chromosomal regions associated with resistance to *Aspergillus* ear rot and aflatoxins. RFLP analyses were performed on approximately 100 $F_3$ families derived from crosses of LB31 x B73 and 75-R001 x B73. The $F_3$ families were also grown in replicated field trials, inoculated, and evaluated for ear rots and aflatoxin levels. Specific chromosomal regions of LB31 and 75-R001 have been identified as significantly associated with lower ear rot and lower aflatoxin levels. Some of the specific chromosomal associations identified with analysis of variance are:

| | |
|---|---|
| ear rot, LB31 - 1L, 2S, 2L, 7L, 8L, 9S | 75-R001 -1S, 4S, 9S |
| aflatoxins, LB31 - 1L, 2L, 8L, 9S | 75-R001 - 4S |

Multiple regression analyses were performed to help reduce the chance of identifying false positive and false negative associations. These analyses identify the following chromosomal regions as having the highest probability of being associated with ear rot and aflatoxin levels:

| | |
|---|---|
| ear rot, LB31 - 7L, 9S | 75-R001 - 4S |
| aflatoxins, LB31 - 1L, 2S, 9S | 75-R001 - 1L |

Noteworthy was that we identified chromosome regions in B73 associated with resistance to ear rot and aflatoxins. This finding is consistent with the observation of transgressive segregation in populations derived from different sources of resistance. We also identified heterozygote marker classes in the $F_3$ mapping populations that had lower ear rot and aflatoxin means than either of the parental marker class means. This observation is consistent with the finding of superior performance of $F_1$ hybrids for ear rot and aflatoxins in comparison to either inbred parents.

Cluster analysis was performed on the RFLP genotypes of eight of the original promising sources of resistance, including LB31 and 75-R001. This analysis revealed that LB31 and 75-R001 are very dissimilar from Mo17 and B73, with LB31 more similar to Mo17 and 75-R001 in a very distant cluster. The analysis indicated that all eight sources of resistance were very dissimilar. This finding increases the possibility that there may be different sources of genetic resistance in these lines because they apparently do not come from one or a few common genetic backgrounds.

1. Campbell, K.W., and White, D.G., Toman, J., and Rocheford, T.R. 1993. Sources of resistance in $F_1$ corn hybrids to ear rot caused by *Aspegillus flavus*. Plant Dis. 77:1169.

2. Campbell, K.W., and White, D.G. 1994. An inoculation device to evaluate maize for resistance to ear rot and aflatoxin production by *Aspergillus flavus*. Plant Dis. 78:778-781.

POSTER

# Detecting Corn Germplasm with Resistance to Aflatoxin Production in Kernel Extracts

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Resistance to aflatoxin production is a desired trait in those crops where aflatoxin has become a problem. In corn there have been several instances of germplasm being developed with resistance to fungal development on the grain, but there are no examples of elite genotypes that inhibit aflatoxin production in spite of being infected by the fungus *Aspergillus flavus.* In most instances, organic inhibitors of aflatoxin production have been general inhibitors of fungus growth. Noteable exceptions are extracts of soybean, neem, cottonseed, and coffee, which appear to inhibit aflatoxin production without affecting growth of *A. flavus*.

Hexane extracts of corn seed appear to inhibit aflatoxin synthesis with no apparent inhibition of growth of *A. flavus*. This may be due to some constitutive inhibitor in corn grain, and thereby a trait that can be enhanced in corn germplasm, possibly through mutagenesis. During the prior 5 months a research project was initiated to: 1)screen mutagenized families of elite corn inbreds by laboratory evaluation for inhibition of aflatoxin production by *A. flavus,* 2) increase aflatoxin suppressive germplasm and determine the heritability of the aflatoxin inhibition trait, and 3) evaluate germplasm for inhibition of aflatoxin formation in field grown corn.

A bioassay procedure was developed to select for mutants that produce high levels of inhibitors of aflatoxin synthesis. The procedure involves adding hexane extracts to an agar medium that will allow for quantitative spectrofluorometric measurements of aflatoxin production after seeding the agar with *A. flavus*. Inbred lines tested by the procedure were highly significantly different in their possession of both stimulators and inhibitors of aflatoxin synthesis. Kernels from 3410 M3 progeny of 3410 mutagenized families of B73 and A632 were assayed and 13 families were selected for reproduction in the field in 1994. This germplasm was selected for screening because of a very high frequency of mutations, approximately one per thousand per locus. Screening has continued in the M3 germplasm of B73 and will continue through the winter on this germplasm and M3 families of A632. Selfed progeny from the 13 families planted in the field in 1994 will be bioassayed for inhibition of aflatoxin formation. Where adequate seed is available, the selfs and M3 families that appear to possess aflatoxin inhibitory properties will be analyzed for frequency of the trait in the material. The 13 families were also backcrossed to the nonmutagenized parent and to Mo17, which will allow for some analyses of the heritability of the trait.

* A. D. Wright, the USDA-ARS cooperator left the USDA recently and the temporary USDA-ARS cooperator will be D. R. Buxton.

POSTER

09

# Effect of Pollen Genotype on Kernel Infection by *Aspergillus flavus* and Aflatoxin Contamination of Corn Grain

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When we evaluate corn inbreds or hybrids for either kernel infection by *Aspergillus flavus* or aflatoxin contamination, we usually grow many genotypes together in the field where they freely intermate. Thus, kernels that we eventually assay will have been produced as a result of fertilization by pollen from many different genotypes. This only adds to the variability that is already present within a kernel which has genetically different tissues: the maternally derived pericarp, the triploid endosperm, and the diploid embryo. An immediate expression of pollen-parent effects, or xenia, has been reported for several grain characteristics. Because *A. flavus* infection and aflatoxin contamination are grain characteristics, they might be affected by the pollen parent as well as the seed parent. The objective of this study was to determine whether or not the genotype of the pollen has an effect on percent kernel infection by *A. flavus* or aflatoxin contamination of the grain. Two resistant, Mp313E and Mp420, and two susceptible, Mp68:616 and SC212M, inbreds were chosen for this study. In separate plots in a replicated test, each inbred was pollinated by itself and each of the other inbreds. About a week after pollination, each pollinated ear was inoculated with *A. flavus* inoculum. Ears were harvested some 63 days after midsilk, dried, and shelled. A sample of 390 kernels was surface sterilized and plated in pitre plates, incubated for seven days, and the number of kernels infected by *A. flavus* determined. The remaining grain from each plot was finely ground and assayed for amount of aflatoxin. As would be expected because the inbreds were selected for resistance or susceptibility, the analysis of the data indicated that female effect were large and significant. Crosses with Mp313E, Mp420, Mp68:616, and SC212M as females had 3.7, 4.6, 10.0, and 17.8% kernel infection by *A. flavus* and 84, 342, 489, and 8569 ppb aflatoxin, respectively. Based on the analysis of variance, male effects were not significant indicating that xenia effects were not present for percent kernel infection or aflatoxin contamination of the grain. Thus, studies to identify and/or develop resistant genotypes can be conducted in a field where many genotypes are growing without the need to control the source of pollen by hand pollination of ears.

# BREEDING FOR RESISTANCE TO AFLATOXIN CONTAMINATION IN ALMOND

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**Project Summary for 1994.**

Nearly all North American production of almonds occurs in California. Almond is an economically important crop due to the high value of the raw and processed product and because approximately 60% of the $650 million crop is exported, mainly to Japan and western Europe. Very low to zero tolerance to aflatoxin (mainly from A. flavus and to a lesser degree A. parasiticus) exist in these markets.

We have shown that almond is unique among the crops known to be vulnerable to preharvest aflatoxin contamination in that vulnerability is largely confined to a short period of fruit ripening between hull and endocarp splitting when the nut is first exposed, and the subsequent field drying of enclosed seed to moisture contents of approx. 7% which effectively arrests further fungal infection and growth. In all genotypes tested in 1994, the intact seedcoat tissue acted as a barrier to initial fungal infection with virtually all field infections identified being associated with worm damage to the nut by the navel orangeworm (NOW), (which hatch from eggs oviposited on the fruit surface and feed on the fruit exocarp before invading the kernel through pre-existing gaps in the endocarp). These results continue to support three possible sites for disease control (a) non-preference and/or antibiosis of exocarp tissue to NOW oviposition and initial larval development, (b) a complete endocarp seal to prevent worm or fungal access to mature nut meats, and (c) seedcoat barriers to larval infestation and/or fungal infection.

Evidence for exocarp antibiosis was obtained from lab and field studies where significantly lower NOW infestation rates were observed in specific genotypes, particularly 'Mission', 'Peerless', and 'Ballico'. Complete freedom from fungal infection of almond seed continued to be observed in genotypes tested which possessed a completely sealed endocarp, suggesting this goal as the most promising resistance breeding strategy. A gas-flowmeter was modified to allow accurate quantification of degree of suture seal of genotypes in the screening program resulting in the further development of a accurate, rapid assay using 'feeler gauge' endocarp probes. Using these procedures it was shown that even fairly well sealed nuts were vulnerable to suture seal breakdown if suture lignification occurred relatively late during secondary mesocarp growth. Critical components of endocarp seal were thus identified as both the quality and timing of lignification at the endocarp suture seal. Using these criteria, several promising breeding lines were selected for continued study, including an interspecific backcross from Prunus argentea (F10D,3-4) which demonstrates very good shell seal leading to apparent freedom from preharvest seed infestation and infection, yet with a very-high crack-out ratio (approx. 80%).

Gradziel, T.M. and D.E. Kester. Breeding for resistance to aflatoxin contamination in almond. (in-press). Acta. Hort.1

*Summary of Panel Discussion: Can We Identify and Utilize Aflatoxin Resistance in Germplasm?*

Panel Members: D. White, D. Wicklow, C. Holbrook, D. Wilson, T. Gradziel, and T. Rocheford.

The panel was unanimous in their opinion that we have the capability of identifying germplasm resistant to *Aspergillus flavus* and aflatoxin. The major concerns at this point are: 1) can we utilize this resistance to improve agronomically acceptable varieties, and 2) what level of resistance is required.

The breeding program dealing with Almond is concentrating on incorporation of inner endocarp and outer mesocarp (hull) barriers to worm infestation as well as seed coat barriers to fungal infection, into cultivars with good horticultural and yield performance. They have identified resistance and are moving the resistance into acceptable varieties through hybridization and potentially through bycrossing. Indications are at this time that they will be successful in producing varieties that are horticulturally acceptable and have high levels of resistance to insect damage, therefore, high levels of resistance to *A. flavus* and aflatoxin contamination.

The goal of peanut research has been to reduce preharvest aflatoxin contamination by 90% of the aflatoxin found with the widely used variety Florunner. A large number of peanut varieties in a germplasm collection have been examined for resistance and promising lines have been identified for further examination. Peanut lines with drought tolerance have also been examined and six of these lines exhibited at least a 90% reduction in preharvest aflatoxin contamination compared to Florunner. Another promising area is with peanut lines that have reduced linolenic acid content. Previous research indicates that peanut lines with lower linolenic acid content are resistant to aflatoxin production. Reduced linolenic cultivars grown in Yuma, Arizona and Tifton, Georgia exhibited aflatoxin levels less than 50% of the level observed in Florunner. The low linolenic acid lines are very close to being released and could serve as a basis for aflatoxin resistance in the next few years. Additionally, resistant peanut lines from the germplasm collection could be crossed with low linolenic acid genotypes producing even higher levels of resistance.

Several sources of resistance to infection by *A. flavus* and aflatoxin production have been identified in corn and have been tested by several researchers using completely different techniques. Most of the sources of resistance have characteristics that are not agronomically acceptable and the resistance must be back-crossed into agronomically acceptable inbred lines. This does not seem to be an impossible task, however, it will require some time. It will be important to understand the heritance of resistance and to be able to utilize RFLP markers for tracking resistance in back-crossing. In two different populations, RFLP markers for resistance to ear rot and for resistance to aflatoxin have been identified. It appears that resistance to aflatoxin formation in the absence of resistance to ear rot resistance may exist. In other words, there may be functional resistance that impedes the fungus' ability to produce toxin and does not necessarily reduce fungal growth. Resistance from several different sources with different genes (mechanisms) for resistance could be crossed into elite inbreds resulting in higher levels of resistance.

There was also no consensus as to what level of resistance is required. Panel members agreed that immunity to *Aspergillus flavus* and aflatoxin may not exist. It does appear, however, that varieties can be produced that will be resistant to the fungus and/or aflatoxin formation in most years. It is also important to note that only a few individuals are evaluating germplasm for resistance and it is likely that numerous additional sources of resistance exist. We have only begun to exploit natural variation for resistance to aflatoxin.

# CROP RESISTANCE - GENETIC ENGINEERING

**Development of Gene Delivery Systems Capable of Introducing *Aspergillus flavus*-Resistance Genes into Peanuts.**

Z. Li, M. Cheng, and J.W. Demski*, Dept. of Plant Pathology, Univ. of Georgia, and R. L. Jarret, USDA-ARS Genetic Resources Unit, Georgia Station, Griffin, Georgia 30223.

An efficient protocol for protoplast-mediated transformation of cultivated peanut (*Arachis hypogaea*) and a wild peanut (*A. paraguariensis*), using electroporation, has been successfully developed. Co-cultivation of protoplasts isolated either from immature cotyledons of cultivated peanut, or from young leaves of *A. paraguariensis*, with peanut nurse cells resulted in formation of callus colonies. After transfer to a regeneration medium, up to 80% of protoplast-derived colonies of cultivated peanut differentiated. Multiple shoots developed from differentiating colonies within a period of two months. Protoplast-derived plants of cultivated peanut, grown to maturity in the greenhouse, were fertile. A poration medium containing biodegradable buffers glycine and glycylglycine was developed to improve direct gene transfer into peanut protoplasts using electroporation. Transformation efficiency, as determined by a transient GUS expression analysis, was increased by 8- to 430-fold using the newly-devised poration medium, when compared to other commonly-used poration media containing stable, non-metabolizable buffers. Kanamycin-resistant colonies have been obtained from protoplasts of both cultivated and wild peanuts after electroporation with the plasmid pBI426 containing the GUS gene and the NPTII kanamycin-resistance gene. DNA analysis using PCR and Southern blot hybridization confirmed the integration of multiple copies of intact transgenes into the peanut genome. Transgenic shoots have been regenerated from transformed callus colonies and are being rooted for further evaluation. Using this protoplast-mediated transformation system, a number of virus coat protein genes, under the control of a highly effective promoter for gene expression in peanut cells, are being introduced into protoplasts of several major US peanut cultivars for the induction of virus resistance. This transformation system should be equally useful for the delivery of anti-fugal genes, that may confer resistance to *A. flavus*, into peanut.

*Agrobacterium*-mediated transformation of peanut leaflets was also achieved. By using a virulent strain EHA105 harboring a binary vector pBI121 containing both the GUS gene and the NPTII gene, a large number of primary putatively transgenic plants, as well as their progeny, have been obtained. GUS expression was consistently detected in most of the regenerated plants. DNA analysis is being conducted to confirm the integration of transgenes in these plants.

## PROGRESS IN THE DEVELOPMENT OF TRANSGENIC PEANUT WITH ENHANCED RESISTANCE TO FUNGI

Arthur Weissinger[1], Lori Urban[1], Rebecca Cade[1], Kim Sampson[1], Gary Payne[2], T. E. Cleveland[3], Peggy Ozias-Akins[4], and Mike Adang[5] (Departments of [1]Crop Science and [2]Plant Pathology, N.C. State University, Raleigh, NC, [3]USDA-SRRC, New Orleans, LA, [4]Department of Horticulture, University of Georgia Coastal Plain Experiment Station, Tifton, GA., [5]Department of Entomology, University of Georgia, Athens, GA)

During the past year, we have focused on improvement of peanut transformation technology, and development of systems for testing the efficacy of antifungal genes, two major areas critical to the development of transgenic peanut with enhanced fungal resistance.

The transformation protocol developed previously (Ozias-Akins et al., 1993. Plant Science 93:185-194.) produces transgenic lines reliably, and in acceptable numbers, although it is very time consuming. We have recovered a limited number of $R_1$ progeny from transformants of cv. Toalson carrying a transgene encoding hygromycin phosphotransferase (HPT). Analysis of these progeny by PCR demonstrated transmission of HPT to the progeny. Because primary transgenics exhibited a high level of barrenness, we also conducted a limited cytogenetic analysis of primary transformants and selected $R_1$ progeny. Results suggest that primary transformants may be aneuploid, but that cultured control plants and R1 progeny possess the normal chromosome complement (2n=40). Because the protracted selection and regeneration used in production of transformants may be responsible for shifts in ploidy, selection in liquid suspensions has been eliminated, and regeneration significantly shortened. Plants derived through this process have been moved into the greenhouse, but molecular analysis is not yet complete. We also have initiated experiments to define *Agrobacterium*-based protocols for peanut transformation. We have confirmed earlier reports that strain EHA 105 infects peanut tissue, but we have not yet recovered transgenic plants.

We have also developed protocols for testing the efficacy of antifungal gene products *in vitro* and *in vivo*, and have demonstrated the efficacy of three lytic peptides against *A. flavus*. Cecropin analogs were obtained through collaboration with Demeter Biotechnologies, Ltd.. The efficacy of these products varies between the different peptides, and also varies with peptide concentration, but all are effective at concentrations above 5 uM. Growth and sporulation are inhibited, and there is strong evidence that at least one of the peptides is lethal to the fungus. Spore germination is also affected at high concentrations. Experiments to produce transgenic peanut carrying cecropin genes are now in progress. We have also developed a procedure in which alien genes can be delivered into cells of mature leaf tissue using *Agrobacterium*. Transgenic callus can be derived by this process in approximately 6 weeks, and the process has been verified by PCR analysis and immunology (Western blot). We anticipate that this system will be useful to identify antifungal genes effective against *A. flavus*, to test them for adverse effect on peanut cells, and to model the effect of alterations which affect gene expression and the secretion or sequestering of gene products. We are currently using this system to transform calli with genes encoding osmotin, a protein known to have antifungal activity.

## GENETIC ENGINEERING OF PEANUT - INSERTION OF FOUR GENES THAT MAY OFFER DISEASE RESISTANCE STRATEGIES

Peggy Ozias-Akins[1], Chong Singsit[1], Ravinder Gill[1], Aiming Wang[1], Mike Adang[2], Bob Lynch[3], Arthur Weissinger[4]
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We have previously developed a transformation protocol that allows the routine introduction of foreign genes into peanut tissues and the regeneration of transgenic plants. Our objectives during the current funding period are to 1) to use our most advanced transformation protocol to introduce putative antifungal genes into peanut; 2) to analyze and conclude *Agrobacterium* transformation experiments already initiated; 3) to determine if a promoter that is subject to modulation by environmental factors conducive to aflatoxin formation might function in stably-transformed peanut plants. All transgenic cell lines recovered in the past year have been from three runner type peanut cultivars, Florunner, Georgia Runner, and MARC-1.

Four genes have been reengineered into a plasmid vector containing a hygromycin resistance gene under the control of the CaMV35S promoter. Our primary emphasis has been on introduction of a synthetic *Bacillus thuringiensis* toxin gene (*cryIA(c)*) obtained from Mike Adang. The CryIA(c) protein has been shown to be very effective in inducing mortality in lesser cornstalk borer larvae. Lesser cornstalk borer (LCB) becomes a serious pest of peanut under hot, dry conditions, the same environment in which aflatoxin production is enhanced. LCB has been shown to carry spores of *A. flavus* to wound sites on peanut pods (Lynch and Wilson, 1991, Peanut Sci. 18:110). It has become apparent that insect damage is highly correlated with high aflatoxin levels not only in peanut, but in cotton, corn, and tree-nuts (1993 USDA Multicrop Aflatoxin Workshop). We have selected 39 hygromycin-resistant cell lines, most of which contain not only the selectable marker gene, but also an intact synthetic Bt gene. CryIA(c) protein has been measured in several of these transformed cell lines and can constitute up to 0.02% of total protein. Several of the cell lines also have been tested by bioassay with first instar LCB larvae and were shown to induce up to 100% mortality. A second bioassay with leaf tissue from transgenic and control plants currently is in progress. We conclude that CryIA(c) will likely be very effective in reducing feeding of LCB on peanut plants which will result in less pod damage, and consequently reduced access by *Aspergillus* spores, as well as reduced population size of LCB.

A second putative antifungal gene is *tap1*, tomato anionic peroxidase, under the control of CaMV35S. Unpublished results (P.E. Kolattukudy, personal communication) indicate that overexpression of this gene in tobacco confers resistance to blue mold, and overexpression in potato reduces susceptibility to *Rhizoctonia solani*. Over 70 transgenic cell lines have been recovered from bombardment experiments and regeneration of whole plants is in progress. All cell lines tested by Southern analysis are positive. Immunoassay for expression of the gene will be carried out by P.E. Kolattukudy on leaf tissues.

The third and fourth genes are tomato spotted wilt virus N gene and a soybean vegetative storage protein promoter fused with beta-glucuronidase. This promoter is inducible by wounding, drought stress, carbohydrates and methyl jasmonate and might be a useful alternative to the CaMV35S for the expression of multiple resistance genes. The TSWV N gene construct was used early in our program to modify the transformation protocol when no antifungal genes were available. We have recovered transgenic cell lines at frequencies comparable to the above described experiments and are continuing to investigate gene integration and expression.

No convincing Southern data could be derived from our *Agrobacterium* transformation experiments; therefore, we conclude that microprojectile bombardment of embryogenic cultures remains the most reliable method for recovering large numbers of transgenic plants.

**Title: Transformation and Regeneration of Cotton to Yield Improved Resistance to A. flavus**

Authors: Caryl A. Chlan, and Lin Junmin (Biology Department, The University of Southwestern Louisiana, Lafayette, LA 70504) and Jeffrey Cary and Thomas E. Cleveland (Southern Regional Research Laboratory, USDA/ARS, New Orleans, LA 70179)

Our goal in this project is to transform and regenerate cotton plants that have been genetically engineered in the laboratory to have increased resistance to Aspergillus flavus. Although the primary product of the cotton industry is cotton fiber, cottonseed is an important by product and its monetary value as animal feed is affected by aflatoxin levels. Conventional control measures have not been effective, and strains of cotton with natural resistance to A. flavus are not available. Genetic engineering of cotton using molecular techniques is a logical approach to this problem.

We have primarily focused on using the biolistic method to transform cotton meristem tissues. After bombardment, these tissues are incubated on selective media to encourage growth of transformed cells into plants. We have continued to optimize the biolistic gene delivery system to obtain the greatest numbers of transformed tissues. Initially, we prepared our meristem sections from sterilized cottonseed that had been incubated for 24 to 48 hours at 30°C. However, more viable sections are observed post bombardment if sections are not held more than a few hours post dissection and prior to bombardment. Optimal bombardment conditions include the use of DNA coated 1.6 micron gold particles accelerated by pressures of 1300 or 1550 psi at tissues a distance of 6 cm. Transformation efficiency is also increased if the same tissue is bombarded twice (either both times at a distance of 6 cm, or once at 6 cm then once at 3 cm).

We have developed an appropriate system for the regeneration of transformed tissues post bombardment. First, tissue sections are maintained on McCown's media in the presence of Kanamycin at 50 mg/L. This level of selection kills non-transformed tissues, but does not significantly retard the growth of transformed tissues. In about 4 weeks, if several leaves, and at least two roots have formed in this media, then the plants are transferred to a modified MS media supplemented with hormones to favor more extensive rooting. When the root system has sufficiently developed to support the growth of the plant (4-6 weeks), plants are transplanted to a mixture of top soil:sand:peat (1:1:1). The plantlets are gradually hardened off over a period of 5-7 days and watered with 1/4 strength Hoagland's Solution.

We have used two methods to determine if plants that root in the presence of kanamycin are transgenic. First, leaf extracts were tested for neomycin phosphotransferase activity using an in vitro kinase assay. Using this method, 7 plants were confirmed as transgenic. However, we decided a more economical screening method would include western blot analysis of leaf extracts to identify proteins that cross react with NPT II antibodies. Using this method, we have identified 31 transgenic cotton plants. Each of these plants has been bombarded with DNA that encodes NPT II and a potential anti-Aspergillus flavus activity - either osmotin, chitinase, polygalacturonidase inhibitor protein, proteinase inhibitor, or a chitinase/glucanase dual construct. We have 8 transgenic cotton plants that should also contain a chitinase gene, 8 PGIP gene plants, and 15 that should also contain an osmotin gene. We have 18 rooting tissue sections that were transformed with the protease inhibitor. Tissues bombarded with the dual chitinase/glucanase construct are still developing. To determine if our transgenic plants also contain the potential anti-pathogen genes, we are screening DNA extracted from leaf tissue. After we have identified plants that contain the genes to enhance resistance to Aspergillus flavus, plant extracts will be tested for their ability to inhibit the pathogen.

**Construction of Transformation Vectors Expressing Resistance Genes to A. flavus in Cotton.**
J. W. Cary, A. J. Delucca, and T. E. Cleveland, USDA-ARS, SRRC, New Orleans, LA; C. Chlan and J. Lin, University of Southwestern Louisiana, Lafayette, LA.

We report on our continued progress on the development of transgenic cotton plants capable of resisting invasion by *A. flavus* and hence contamination by aflatoxins. The success of this project will depend in large part on two major areas of research. First, it is essential to identify factors that demonstrate antifungal activity or inhibition of toxin synthesis and then clone or synthesize the gene(s) responsible for their production. Secondly, these genes must be engineered into vectors that will allow for their optimal temporal and spatial expression once they have been transformed into cotton tissues.

A number of candidate antifungal proteins and peptides have been identified and when possible their *in vitro* efficacy has been assayed prior to construction and transformation of vectors harboring the gene responsible for their synthesis. Antifungal proteins tested to date include a bacterial endo-chitinase and a tobacco osmotin. *In vitro* assay of chitinase activity has demonstrated inhibition due to lysis of *A. flavus* mycelia. In addition a crude preparation of polygalacturonase inhibitor protein (PGIP) from *Phaseolus vulgaris* has shown low levels of inhibition of endo-PG activity in *A. flavus*. This may be useful in slowing the progression of the fungus during invasion of the plant by interfering with it's ability to hydrolyze and gain access to plant cell wall components. We have also performed *in vitro* spore germination inhibition assays with the tobacco antifungal protein, osmotin, and the small lytic peptide of porcine origin, cecropin. Treatment of *A. flavus* spores with osmotin (10μM) resulted in no delay of *A. flavus* spore germination. Treatment of spores with cecropin (125 μM), a small (MW= 4 kDa) lytic peptide, delayed spore germination for up to 6 h.

The genes encoding the above mentioned proteins/peptides have either been engineered into plant expression vectors or will soon be once vectors for their delivery into cotton have been constructed or made available. We have successfully transformed into cotton tissues, via *Agrobacterium* or biolistic techniques, constructs harboring the genes encoding PGIP, osmotin, and basic bean chitinase. To date, successful transformation has been based on assay for the kanamycin resistance marker present on the vector (KYLX 7.1 utilizing the CaMV 35S promoter). As enough tissue becomes available we will assay (Southern, Northern, and Western blotting) for the presence and expression of the antifungal gene. Constructs harboring synthetic genes encoding the lytic peptides D5-C and D4E-1 (Demeter Biotechnologies, Ltd.) will also be engineered for optimum expression in cotton. This will necessitate the use of gene fusions in many cases (i.e., ubiquitin, cotton seed storage protein promoters fused to functional signal peptide sequences for secretion into the extracellular space). Intracellular localization of these peptides may be achieved by excision of the signal sequence or addition of an endoplasmic reticulum retention signal sequence at the carboxy-terminus of the synthetic gene. Subsequent bioassay of transgenic plants will be performed to study the production and efficacy of all antfungal gene products which will aid in development of improved antifungal gene constructs if needed.

ANTIFUNGAL ACTIVITY OF OSMOTIN ON VARIOUS FUNGI IN VITRO.

L.R. Todd, M. Paino D'Urzo, P.M. Hasegawa, and R.A. Bressan, Horticulture Department, Purdue University, West Lafayette, IN 47907.

Various fungi were tested for sensitivity *in vitro* to osmotin, a group 5 pathogenesis-related (PR) protein that accumulates in tobacco under osmotic stress. Fungi were subcultured to fresh potato dextrose medium and allowed to grow to a radius of 2 cm. Sterile filterpaper disks, each loaded with one of the following: water, bovine serum albumin (BSA) (100 g), or osmotin (30, 60 or 100 g) were placed outside the leading edge of the colony. After incubation at room temperature for 3 days, a zone of growth inhibition was evident surrounding the disks containing 30, 60 or 100 g osmotin in *Verticillium dahliae, Fusarium oxysporum* f. sp. *dianthi*, *F. oxysporum* f. sp. *lycopersici, Bipolaris maydis* and *Kabatiella zeae*. A growth inhibition zone was evident around disks containing 60 or 100 g osmotin in *Diplodia maydis, Phytophthora infestans* and *Phytophthora parasitica* var. *nicotiana* and around disks containing 100 g osmotin in *Magnaporthe grisea, Botrytis cinerea*, *Colletotrichum graminicola* and *C. gloeosporioides*. No effect of osmotin was seen on *Rhizoctonia solani* at these concentrations. Neither water nor BSA caused growth inhibition of any of the fungi tested. Disks containing 100 g of thaumatin, a PR-5 protein with 55% homology to osmotin, were found to have no activity when tested on *Fusarium oxysporum* f. sp. *dianthi, F. oxysporum* f. sp. *lycopersici, Verticillium dahliae* or *Rhizoctonia solani*. Osmotin was also effective against several of these fungi and *Aspergillus flavus* in spore germination and viability assays. These results indicate that osmotin inhibits the growth of a wide range of fungi *in vitro*.

# PROGRESS IN ENGINEERING WALNUTS FOR RESISTANCE TO ASPERGILLUS FLAVUS

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Our goal is to reduce aflatoxin contamination in tree nuts by genetically engineering the host plant to resist infection by *Aspergillus flavus*. Genetic improvement is a long-term approach to controlling aflatoxin contamination in affected crops. Classical breeding for resistance to the causal agent or its vector can be greatly enhanced by the incorporation of biotechnological approaches, such as introduction of useful foreign genes into breeding lines. For the tree nut crops, walnuts can serve as a model system for this technology because transformation and regeneration of transgenic plants has been accomplished and is relatively routine. The same system has been applied successfully to pecan. In addition, the initial regenerant in the walnut transformation system, the transgenic somatic embryo, which multiplies readily and is similar to the walnut kernel, can be used to assess the response of the causal fungus to the introduced genes, in vitro. This research summary describes our progress in 1994.

Our objectives were to continue to test the efficacy of chitin binding genes for reducing infection of embryos by *A. flavus*; to select and multiply promising clones for field trials, and to investigate the potential of other genes for controlling *A. flavus*.

A new protocol was followed to test the efficacy of the chitin binding genes. Three transgenic embryo lines containing the lectin genes of hevein (HEV 1-3), nettle (N 2-2), or barley (B 2-5) were compared with an untransformed control (SU2) and a transformed control (Pmon 3-6). Individual embryos ($n=10$/line X 2 reps) were placed on moistened filter paper in the inner wells of 24 well multi-well plates and inoculated with about 60 spores each of *A. flavus* strain A296 provided by Themis Michailides. Plates were incubated at 20C for 5 days to allow the mycelium to grow and then incubated at 28C for 24 h to induce sporulation. Spore counts were taken and spores/g fresh weight were calculated. Sporulation was significantly ($p<.01$) reduced when compared to the controls on embryos containing the barley gene. The effect was less pronounced ($.05<p<.1$) on the other embryo lines. All three lines have been selected for field trials.

Eight new transgenic embryo lines containing a systemic acquired resistance gene (SAR 8.2) were recovered from transformation of walnut embryo line SU2 with the binary vector pDU92.213 in *Agrobacterium* strains EHA101 and C58. These lines will be bioassayed using the above procedure. Several other genes were identified which may provide protection from *A flavus*. These include the gene for the polygalacturonase inhibition protein (PGIP) which has been combined with GUS in plasmids pDU94.0928 and pUD94.0935 and inserted into EHA101 and C58 in preparation for transformation. Three genes encoding lytic peptides are also under investigation and plasmids are being constructed to contain the GUS gene.

Control of aflatoxin contamination through enhancement of genes/traits suppressing fungal growth and aflatoxin. T. E. Cleveland, J. W. Cary, R. L. Brown, A. J. Delucca, D. Bhatnagar, SRRC, ARS, USDA, New Orleans, LA; C. A. Chlan, J. Lin, Univ. Southwestern LA, Lafayette, LA.; G. A. Payne, R. Boston, Ashwin Mehta, N. C. State Univ. Raleigh, N. C.; J. S. Russin, B. Guo, Louisiana State Univ., Baton Rouge, LA.

Biotechnological solutions to the aflatoxin problem are being developed involving the use of inhibitors of fungal growth and/or the aflatoxin biosynthetic pathway. A. flavus strains transformed with the ß-D-glucuronidase (GUS) reporter gene linked to fungal growth- and aflatoxin-specific genes, have been constructed. An A. flavus strain transformed with the growth specific (ß-tubulin) reporter gene construct was used to inoculate endosperm-wounded corn kernels in the laboratory and to assess resistance to infection by the fungus in kernel tissues. Some corn inbreds showed remarkable resistance to fungal infection and growth in kernel tissues. In some corn inbreds, high levels of potentially antifungal proteins, ribosomal inactivating proteins (RIP), were correlated with low levels of fungal invasion of the endosperm (the site of RIP gene expression) by the GUS gene-containing tester fungus. Another corn variety (MASgk) appeared to support substantial fungal growth but relatively much less (about 6%) aflatoxin production compared to a "susceptible" control, thus indicating the presence of kernel factors modulating toxin biosynthesis. Reporter gene constructs under the control of specific aflatoxin gene promoters will be used to assess living kernels and seed compartments for presence of compounds modulating levels of aflatoxin biosynthesis. Native kernel traits that minimize fungal infection and aflatoxin biosynthesis could be useful as selectable markers in corn breeding to reduce aflatoxin contamination.

Antifungal genes are being cloned from plants and microbes for genetic engineering of cotton for resistance to aflatoxin contamination. The genes for candidate antifungal proteins and peptides have been identified and when possible their in vitro efficacy assayed prior to construction and transformation of cotton with vectors harboring the gene responsible for their synthesis. Some cecropin-based peptides delayed spore germination of A. flavus; cecropin (125 uM) from a mammalian source delayed spore germination of this fungus several hours relative to untreated controls. Small lytic peptides designed based on the cecropin mode of action (Demeter Biotechnologies Incorporated) were even more potent inhibitors of A. flavus in bioassays. We have successfully transformed cotton via Agrobacterium or biolistic techniques and stably maintained constructs harboring the genes encoding potentially antifungal proteins (eg. the basic bean chitinase) in cotton tissue. Synthetic genes are being constructed which code for small lytic peptides to be used in cotton transformation. Successful transformation has been based on assay for the kanamycin resistance marker present on the vector (KYLX 7.1 utilizing the CaMV 35S promoter).

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POSTER

2.

**MECHANISMS OF MAIZE KERNEL RESISTANCE AGAINST *ASPERGILLUS FLAVUS* AND/OR AFLATOXIN CONTAMINATION.**

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In an effort to gain a greater understanding of the relationship between fungal growth and aflatoxin contamination, and to learn more about the mode of action of resistance mechanisms being expressed in different maize genotypes, fungal growth and aflatoxin contamination in kernels of the GT-MAS:gk population were quantitated and compared, in 4 separate experiments, to levels in kernels of a susceptible Pioneer line (3192) and 3 inbreds. The GT-MAS:gk (MAS) population and inbred Tex6 have shown substantial resistance to aflatoxin contamination by Aspergillus flavus in field trials (3,6). All entries tested (minus Pioneer 3192) have demonstrated resistance to contamination in kernel screening assays (1,2,4).

Kernels to be tested were inoculated with an A. flavus aflatoxin-producing strain containing the Escherichia coli B-D-glucuronidase (GUS) reporter gene linked to an A. flavus B-tubulin gene promoter. Prior to inoculation, kernels were pin wounded through the pericarp to the endosperm, pin wounded in the embryo region or left intact. Seven days after inoculation, B-glucuronidase activity in kernels was quantified using a fluorogenic assay and aflatoxins in these same kernels were analysed.

Results show that resistance to aflatoxin contamination is demonstrated in inoculated intact kernels of MAS and all 3 inbreds tested (CI2, MI82 and Tex6). When the pericarp barrier is penetrated, resistance is maintained in the 3 inbred lines, but is maintained in MAS to a much lesser extent, and not in all experiments. The MAS population has demonstrated resistance to aflatoxin contamination in endosperm-wounded kernels in previous studies, however the magnitude of aflatoxin supported by kernels after the pericarp is breached exhibits considerable inter-test variation (1,2,4). Wounding the kernel embryo greatly increased aflatoxin levels in all entries except MI82.

Lower aflatoxin levels in endosperm-wounded kernels and lower total levels for MAS occurred even though MAS kernels supported fungal growth equal to that supported by Pioneer 3192 kernels. Lower toxin levels in endosperm-wounded kernels of CI2 correlate with lower fungal growth in kernels of this genotype. For entry MI82 (endosperm-wounded kernels), lower aflatoxin B1 quantities are associated with both lower growth and lower aflatoxin biosynthesis per unit of fungal growth. The same is true for endosperm-wounded kernels of Tex6.

The large increases in both fungal growth and aflatoxin B1 contamination seen in embryo-wounded kernels of MAS, CI2, Tex6 and Pioneer 3192 genotypes, demonstrate again that the embryo is generally superior to the endosperm, as a substrate for fungal growth and aflatoxin production (1,5). Previous studies have also demonstrated that embryo (and aleurone) tissue is colonized prior to endosperm tissue in susceptible and resistant (where colonization occurred) kernels, whether intact or wounded (2). Thus, kernel resistance to aflatoxin contamination, may be associated with the ability of kernels to prevent or limit fungal access to the embryo region. In a previous study, kernel ability to resist aflatoxin contamination was demonstrated only in kernels with viable embryos (1). This may point to an active role for embryo tissue, in the initiation and/or regulation of kernel resistance mechanisms.

In the present study, the ability to resist aflatoxin contamination was demonstrated in kernels of entries MAS, CI2, M182 and Tex6. However, the mode of action of the resistance mechanisms conveying this ability may vary among genotypes. All 3 inbreds as well as MAS demonstrated resistance in intact kernels that is probably morphological in nature. A previous study has attributed a portion of MAS kernel resistance to waxes and cutins present in the kernel pericarp (4). Entries CI2, MI82 and Tex6 also supported low aflatoxin levels in kernels where the pericarp had been breached, and where

A. flavus had been given immediate access to the endosperm. The mode of action of internal resistance mechanisms in CI2 endosperm-wounded kernels may be antifungal, however, in entries MI82 and Tex6, aflatoxin biosynthesis appears to be affected as well. The low levels of toxin in embryo-wounded kernels of MI82 may be due to an unfavorable kernel substrate for fungal growth and aflatoxin production rather than to the action of resistance mechanisms. In the present study, MAS kernels wounded in the endosperm, showed resistance to contamination despite supporting fungal growth that was equal to the susceptible control. In this case, antitoxigenic mechanisms may be at work.

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Panel Discussion:**Opportunities and Obstacles in a Genetic Engineering Approach**

Panel Members: Ray Bresson, Caryl A. Chlan (chair), Thomas E. Cleveland, Abhaya Dandekar, Zhijan Li, Peggy Ozias-Akins, Sadik Tuzum, and Arthur Weissenger.

The panel discussion focussed on two general areas. There was some discussion about problems that had been encountered in the regeneration or tissue culture of specific plants. However, it seemed that modified protocols alleviated many of the earlier problems and these modifications have decreased the time necessary for regeneration of transgenic plants. The sentiment was that transformation and regeneration are no longer the rate limiting steps in the generation of genetically engineered cotton, peanuts, or walnut.

Most of the panel discussion centered on the choice of genes to be transformed into plants to confer resistance to Aspergillus flavus. A variety of gene constructs have been introduced into plants. These include genes encoding the following proteins : BT, chitin binding genes, tomato anionic peroxidase, tomato spotted wilt virus nucleoprotein, basic bean chitinase, ribosome inactivating protein (RIP), osmotin, polygalacturonase inhibitor protein (PGIP), lytic peptides, protease inhibitor, and chitinase/glucanase (dual construct). Many of the studies of these plants are incomplete, and data is not yet available concerning the levels of resistance of these plants to pathogens. In at least one instance, it has been shown that transgenic plants have increased resistance to a pathogen (BT gene expression in peanut is effective against the lesser corn stalk borer).

Perhaps the two genes that are currently the most promising to confer resistance to A. flavus encode lytic peptides and osmotin. A number of groups either currently or in the near future will transform walnut, peanut, and cotton with genes that encode lytic peptides. Although in vitro tests appear promising, efficacy against fungal pathogens, and A. flavus in particular, will be dependent on the stability of these peptides, and expression of appropriate amounts in specific plant tissues. Osmotin is a member of the pathogenesis related proteins, and has been shown to be effective in inhibiting the growth of some fungi. At high concentrations, osmotin inhibits A. flavus growth by 30%. Although the mode of action of this molecule is unknown, it is believed that osmotin is involved in the induction of leaks in the cell wall, similar to the formation of a pore. When osmotin is expressed in transgenic plants, it has demonstrated activity against Phytopthora. Several of the groups are transforming plants with osmotin constructs.

It may be that none of these genes alone is sufficient to confer resistance to A. flavus. For this reason, the possibility of introducing cassettes of resistance genes was discussed. It is clear that in many cases, synergy has been demonstrated, and exogenous levels of inorganic molecules may also effect the efficacy of a compound. For example, the anti-fungal effects of osmotin can be negated in the presence of cations. All of these factors may effect the levels of expression required to inhibit A. flavus.

Once appropriate anti-fungal genes have been identified, and used to transform plants, several other issues must be considered. New genetic information should not encode proteins that would be toxic to the host plant or the host that consumes the material. To optimize the efficacy of expressed anti-fungal compounds against A. flavus, tissue specific expression at specific times may be necessary. This may involve the use of tissue specific and/or would inducible promoters in conjunction with anti-fungal structural genes.

# MOLECULAR BIOLOGY

**Genetics of Aflatoxin Biosynthetic Pathway: Utility in Aflatoxin Elimination Strategies**

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Aflatoxins $B_1$ and $B_2$ are secondary metabolites produced by the filamentous fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare that infect corn, cotton, peanut and tree nuts. These compounds are known to be toxic and carcinogenic to animals and present a potential threat to the health of human beings. In order to devise strategies for reducing or eliminating aflatoxin contamination from food and feed, extensive biochemical and genetic studies have been conducted in our laboratory to better understand how and why aflatoxin is synthesized by the fungi. Several pathway genes and one regulatory gene have been cloned in our laboratory:

Characterization of the aflatoxin pathway regulatory gene: Previously, a gene (*aflR*) was isolated from an *Aspergillus parasiticus* cosmid clone whose expression was correlated with expression of several aflatoxin pathway genes. A 637-bp *SmaI/XhoI* fragment of *aflR*, containing the region encoding the $Zn(II)_2$-$Cys_6$ zinc binuclear cluster motif, was subcloned into the expression vector, pET29c, transformed into B21(DE3)pLysS *E. coli* and grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). From the IPTG-induced culture a fusion protein (AFLR1) was obtained with the expected mol. wt. of 32 kDa. AFLR1 was partially purified by chromatography on nickel-nitriloacetic acid-Sepharose 4B resin (NiNTA), which binds to a $His_6$ site introduced into the recombinant protein at the C-terminus. Electrophoretic mobility shift assays (EMSA) were performed with portions of the 5'-upstream regions of the *aflR* promoter region. From these studies, a region in the *aflR* gene 120 bp upstream of the translation start site containing the palindromic sequence TTAGGCCTAA was found to bind specifically to recombinant AFLR.

Cloning and disruption of a polyketide synthetase gene: Using a deletion construct, pXX, we disrupted the aflatoxin pathway in *A. parasiticus* SRRC 2043, resulting in inability to produce aflatoxin intermediates and a major yellow pigment in the transformants. The disruption was attributed to a single crossover, homologous integration event between pXX and the recipient *A. parasiticus* genome at a specific locus, designated *pksA*. Sequence analysis suggests that *pksA* is a homolog of the *Aspergillus nidulans wA* gene, a polyketide synthase gene involved in conidial wall pigment biosynthesis. The conserved β-ketoacyl synthase, actyltransferase and acyl carrier protein domains were present in the deduced amino sequence of *pksA*. No β-ketoacyl reductase and enoyl reductase domains were found, suggesting that *pksA* does not encode catalytic

activities for processing β-carbon similar to those required for long chain fatty acid synthesis. The *pksA* gene and the *nor-1* gene, an aflatoxin pathway gene required for converting norsolorinic acid to averantin, were divergently transcribed from a 1.5 kb intergenic region. We propose that *psKA* is a polyketide synthase gene required for the early steps of aflatoxin biosynthesis.

Cloning of pathway gene, *norA*: A gene (*norA)* involved in an early step of the aflatoxin biosynthetic pathway was isolated from *Aspergillus parasiticus* ATCC 2043 using monoclonal antibody produced against the purified enzyme demonstrating norsolorinic acid (NOR) reductase activity. The antibody was used to screen a lambda-ZAP cDNA library generated from 48 h mycelia of *A. parasiticus*. A clone harboring a plasmid with a cDNA insert of 1.4 kb, designated pNOR43, produced a fusion protein of about 46 kDa as determined by Western blotting. A transcript of about 1.5 kb was detected on Northern blots of total RNA isolated from 48 h mycelia. DNA sequence analysis identified an ORF of 1167 bp encoding a deduced protein of 43 kDa. The deduced polypeptide had an amino acid sequence identity of 48% with an aryl alcohol dehydrogenase from *Phanerochaete chrysosporium*.

Utility in aflatoxin elimination strategies:

(1) We have generated an aflatoxin non-producer that does not make any aflatoxin precursors. That was accomplished by disrupting a critical gene in the early stages of the pathway. This strain can be used as a biocontrol agent.

(2) With the characterization of the regulatory gene we now have the tools for detecting how aflatoxin biosynthesis is turned on and what parameters, environmental or otherwise, are required for the onset of this process.

(3) We now have the information necessary to determine what role the genetic machinery for aflatoxin biosynthesis has in the survival of the fungus. This is very significant because we have observed that the highly competitive natural strains, even though they are aflatoxin non-producers, have the entire biosynthetic machinery intact, whereas the machinery is absent in the non-competitive strains.

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Molecular Genetic Approaches to Preharvest Elimination of Aflatoxin Contamination.

S.H. Liang, N. Mahanti, M. Rarick, F. Trail, D. Wilson, T.S. Wu, R. Zhou, J. Linz, Dept. Food Science and Human Nutrition, Michigan State University.

In the first 3 years of this project 3 genes, nor-1, ver-1, and UVM8, were cloned by complementation of aflatoxin "blocked" mutant strains of A. parasiticus. Each of these genes has been experimentally demonstrated (by gene disruption) to function in Aflatoxin B1 (AFB1) biosynthesis. These three genes were found to be genetically linked in a cluster contained on two cosmids, NorA and NorB. Eleven additional genes (including aflR) were shown to be contained in this cluster and the pattern of their expression suggests that they also may be involved in AFB1 synthesis.

There were 3 specific objectives for the current project (funded 7-1-94 to 6-30-95). Progress on each of these objectives is presented here.

(1) Continue the structure/function analysis of cosmid NorA allowing the construction of a fine structure map of genes located in the AFB1 cluster. The entire chromosomal region contained in cosmid NorA is undergoing nucleotide sequence analysis (in collaboration with USDA New Orleans). Approximately 18 kb of the 36 kb insert in cosmid NorA has been sequenced. A restriction map of an AFB1 gene cluster has been generated in A. flavus (in the USDA New Orleans lab and in G. Payne's lab). The overall order and organization of genes in the clusters from these two species of Aspergillus are similar but the spacing between the genes is not perfectly conserved. Identification of nearly identical putative regulator genes (aflR, G. Payne, A. flavus; aflR Bhatnagar, Payne, Linz et al., A. parasiticus) in both species suggests that certain aspects of regulation of pathway function may be conserved. Of particular interest, a 12 kb region containing ver-1 and aflR was shown to be present in duplicate copies in A. parasiticus SU-1 perhaps helping to explain the relatively high genetic stability of toxin production in A. parasiticus as compared to A. flavus in which single copies of all AFB1 genes studied have been identified. Nucleotide sequence analysis of the ver-1 duplicate gene, called ver-1B, demonstrated that it is probably non-functional (pseudogene) due to the occurrence of an in-frame stop codon near the 5′ end of the gene.

(2A) Complete the functional analysis of 3 genes on cosmid nor-A which appear to function early in the pathway. These genes encode large transcripts (7.0, 7.5, and 6.5 kb) and are located adjacent to the nor-1 gene. The functional disruption of UVM8, a putative polyketide synthetase (PKS) or fatty acid synthetase (FAS), (one of these 3 genes encoding the 7.5 kb transcript), has been achieved (see poster by Mahanti, et al.). Experimental evidence demonstrated that the gene encoding the 7.0 kb transcript also has been disrupted. This gene clearly functions prior to ver-1 in AFB1 synthesis. Preliminary sequence data suggest this gene is a PKS because it has significant identity with the wA gene from Aspergillus nidulans which is a PKS involved in conidial pigment synthesis. No identifiable AFB1 pathway intermediates (visible or UV fluorescing) accumulate in the "knockout" strain suggesting that the gene may function early in AFB1 synthesis. Work on disruption of the gene encoding the 6.5 kb transcript is now underway, but preliminary sequence data show significant sequence identity with FAS1 from Saccharomyces cerevisiae in a functional domain identified as an enoyl reductase.

(2B) Complete the functional analysis of ver-1; The product of this gene is associated with conversion of versicolorin A (VA) to sterigmatocystin (ST) in AFB1 biosynthesis. The protein encoded by ver-1A has been expressed as a Maltose

Binding Protein (MBP) fusion product in E. coli and polyclonal antibodies (PAb) have been raised in rabbits. An enzyme assay for the recombinant protein is under development and the PAb appear to react specifically with the native ver 1A protein in A. parasiticus SU-1, but not in a ver-1 knockout strain. Genetic disruption of ver-1 was complicated because of the ver 1A/ver 1B gene duplication in SU-1. In a recent knockout experiment, preliminary data now suggest that a large region carrying ver-1A and downstream genes (maybe including omt-1) has been duplicated (3 copies of ver-1). Southern hybridization data suggest we have disrupted one of the two copies of ver-1A in one strain and both copies of ver-1A in another. The double knockout strain accumulates versicolorin A and not AFB1 and also does not appear to make the ver-1A protein.

The nor-1 protein has also been expressed as a MBP fusion product in E. coli and PAb raised in rabbits. The nor-1 fusion protein converts norsolorinic acid to averantin in vitro consistent with its proposed NA reductase function. Disruption of this gene was reported last year. Work on the structure and function of nor-1 and ver-1 promoter regions is progressing (see poster by F. Trail). Preliminary data suggest that at least 2 specific protein/DNA interactions are involved in nor-1 promoter regulation.

(3) Construct a genetically stable nontoxigenic isolate of A. parasiticus NRRL 6111 (brn-1, nor-1) for field testing on peanuts (in collaboration with Dr. Richard Cole).

Three independent UVM8 disruption strains Dis-1, 2, and 3 have been generated in A. parasiticus B62 (brn-1, niaD, nor-1) which is an niaD strain derived from A. parasiticus NRRL 6111 (ATCC 24690). These "knockout" strains were shown by ELISA and TLC to produce no detectable AFB1 or AFB1 pathway intermediates (see poster by Mahanti et al.). Strain Dis-3 is most promising because it results from a deletion of part or all of the UVM8 gene and loss of vector sequences. Dis-1, 2, and 3 have been forwarded to Dr. Cole for potential initial testing for growth, competitiveness and possibly for use in biocontrol.

Molecular Approaches to Control Aflatoxin Contamination of Food Sources
1994 Annual Report

G. A. Payne, Department of Plant Pathology, North Carolina State University.

Cooperators: D. Bhatnagar and T. E. Cleveland, USDA/ARS/, Southern Regional Research Center, New Orleans, LA.

The overall goal of my research program is to develop control strategies for the elimination of aflatoxin contamination. Research in my lab has focused on understanding the aflatoxin biosynthetic pathway such that strategies can be developed to disrupt aflatoxin biosynthesis. To achieve this goal we are characterizing genes in the pathway and studying their regulation. Described below is the progress we have made in this research.

**Development of a karyotypic map of *Aspergillus flavus*.**

Currently little is known about *A. flavus* genetics. The fungus has no sexual cycle and, thus, recombination frequencies and linkage group data are difficult to obtain. It does, however, have a well characterized parasexual cycle which has been used to determine the presence of eight linkage groups. The purpose of this research was to develop a karyotypic map and assign linkage group markers to chromosomes of *A. flavus*. Such a map will allow mapping of genes to their respective linkage groups. To accomplish this, we first cloned seven previously mapped auxotrophic genes by genetically complementing strains containing mutated alleles. Second, we separated seven *A. flavus* chromosomes that ranged in size from 7.0 to 2.3 Mb by pulsed-field gel electrophoresis using two independent protocols. Third, we hybridized linkage-specific probes (generated from the cloned parasexually mapped auxotrophic genes) to the chromosomes separated by pulsed-field gel electrophoresis. This allowed us to generate a karyotypic map of *A. flavus* by assigning seven known linkage group markers to individual chromosomal bands. In addition, we assigned five previously unmapped genes to their linkage groups using this karyotypic map. The *benA*, rDNA, *pyrG*, *aflR*, and *adh1* genes were assigned to linkage groups I, II, II, VII, and VII, respectively. The karyotypic map generated in this work allows the assignment of genes to chromosome and eliminates the need for parasexual analysis. Genes cloned from *A. flavus* now may be readily assigned to their linkage groups by their location on a specific chromosome. In addition to facilitate mapping, the ability to separate chromosomes will aid in the cloning of genes. Individual chromosomal bands can be excised, subcloned, and used to generate chromosome specific libraries. Such libraries could facilitate more rapid cloning of genes by chromosomal walking.

**Role of natural antisense in the regulation of *aflR***

We know that *aflR* is required for the expression of the aflatoxin pathway genes. Thus understanding the regulation of this gene is very important. We previously reported the presence of two overlapping, divergent transcripts from this locus. The open reading frames of these two transcripts are 1024 and 1310 bp. The larger transcript (*aflR*) encodes a putative protein containing DNA binding motif. The role of

the second transcript (*aflRas*) in the biosynthesis or regulation of aflatoxin is not known; the transcript does not contain any large open reading frames. We hypothesize that it may be acting as natural antisense to control the expression of the sense strand as has been shown for another fungus, *Dictyostellium discoidium*. The antisense transcript is present in cultures grown under conditions conducive and nonconducive for aflatoxin biosynthesis. To test the hypothesis that the natural antisense can down-regulate aflatoxin biosynthesis, we overexpressed *aflRas* under a constitutive promoter, *gpd* (glyceraldehyde phosphate dehydrogenase). Overexpression of *aflRas* reduced aflatoxin levels 99%. We are currently investigating the promoter region for this transcript.

**Overexpression of *aflR***

We also investigated the effect of overexpression of *aflR* on aflatoxin biosynthesis. We made a construct containing the *gpd* promoter fused to *aflR*. When we expressed this construct in a nonaflatoxin producing strain, we complemented aflatoxin to wild-type levels; however, we have been unable to overexpress aflatoxin. Apparently the regulation is under tight control by one of three possible mechanisms: 1) *aflR* is an effective binding protein and all the promoter sites are bound by normal levels of *aflR*; 2) another factor is involved in binding to the promoter along with *aflR* and this factor is limiting; 3) there is feedback regulation of the pathway independent of the level of *aflR*. We are investigating these possibilities. We found another interesting result when we overexpressed *aflR*. Overexpression of *aflR* led to abnormal development of conidial heads in *A. flavus* and the accumulation of a brown pigment in the transformed strain. These results indicate that there may be a link between aflatoxin biosynthesis and sporulation.

**Development of gene expression systems to study factors involved in resistance and aflatoxin accumulation**

We have begun using the information gained from our pathway studies to develop gene expression systems that may be used to identify inhibitory compounds or resistant genotypes. One system is based on a β-tubulin::GUS construct. This construct developed in our lab has been used in an assay developed by Dr. Robert Brown (USDA/ARS/ SRRC) to monitor the growth of the fungus in corn kernels. We have also made two other constructs, *nor1*::GUS, and *ver1*::GUS. Dr. Charles Woloshuk (Purdue University) has used these constructs to develop an assay to identify stimulatory and inhibitory compounds in corn kernels. We also developed a construct to assay for a potential inhibitory compound to the fungus. We fused the *ver1* promoter with the gene coding for the corn RIP (ribosome inactivating protein). Expression of this construct within *A. flavus* inhibited the growth of the fungus. These results lead to further testing of corn kernels differing in their content of RIP. These studies were done by Dr. T. E. Cleveland (USDA/ARS/SRRC) using the β-tublin::GUS assay. Early results indicate that RIP is inhibitory to *A. flavus* in corn seeds. These results are exciting because results from tests of fungal mycelium or fungal protoplasts indicated that RIP was not active. We hypothesize that RIP is not able to get into the fungus under conditions normally used to test for antifungal activity, and that our assay system circumvented this obstacle.

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Foutz, K. R., C. P. Woloshuk, and G. A. Payne. Cloning and assignment of linkage group loci to a karyotypic map of the filamentous fungus *Aspergillus flavus*. Submitted to Genetics.

Yu, J., P.-K. Chang, M. Wright, D. Bhatnagar, T. E. Cleveland, G. A. Payne, and J. E. Linz. Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. Submitted to Appl. Environ. Microbiology

# IMMUNOCHEMICAL STUDIES OF THE ENZYMES OF AFLATOXIN BIOSYNTHESIS

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Attempts were made to produce both monoclonal (Mab) and polyclonal (Pab) antibodies against several key enzymes involved in aflatoxin (AF) biosynthesis, i.e. sterigmatocystin (ST) methyltransferase (ST-MTFase), norsolorinic acid reductase (NSR), Ver-1 and polyketide synthetase (PKS). The objectives for these studies are: (1) to use the immunoaffinity method for purification of the enzymes, (2) to further our understanding on the structure and function of these enzymes as well as their relationship with AFB1 formation, (3) immunochemical neutralization of the enzymes, and (4) to clone genes of these enzymes in collaboration with USDA scientists by supplying them with immunochemical reagents for enzyme characterization. During 1994, much of our effort was devoted to the production and characterization of antibodies against ST-MTFase, NSR and Ver-1. Effective enzyme-linked immunosorbent assays (ELISA) were established for these three enzymes and subsquently used in a study elucidating the role of these enzymes for AFB1 formation. Progress of this year's work follows:

(A) Studies on ST-methyltransferase: An effective new spectrofluorometric method for ST-MTFase assay was established last year. During the current year, the correlation between the enzyme activity and an Mab-based ELISA for ST-MTFase was made. We found a good correlation between enzymatic activity as determined by the fluorometric method and a newly established Mab-based ELISA. The ELISA method was more sensitive than the enzyme assay. Polyclonal antibodies against the 40 kDa ST-MTFase were produced and well characterized last year (Publication 1). The antibodies have also been used successfully for screening the cDNA library in cloning the gene for the enzyme and in monitoring the enzyme produced in *Escherichia coli* in which *omt*-1 gene was expressed (Publication 2).

Efforts were made to produce Mab against ST-MTFase during this year. Two immunogens were used in the immunization. One immunogen (40-42 kDa) was prepared by passing a partially purified enzyme preparation through an immunoaffinity column in which the Pab against the enzyme was conjugated. Another immunogen (46 kDa) was prepared by electroelution from a SDS-PAGE in which a partially purified enzyme preparation was applied to the gel. Analysis of the antiserum from BALB/c mice with ELISA revealed that the antiserum from each group can recognize both the antigens (40-42 kDa and 46kDa). A total of 23 hybridoma cell lines that elicited antibodies against both the 40 and 46 kDa ST-MTFase were obtained. Further re-cloning of the cell lines resulted in 8 stable clones. Among these, 3 clones elicited Mab showing good specificity, primarily against the 46 kDa ST-MTFase, and 5 clones elicited Mab with specificity primarily for the 40 kDa enzyme. Two Mab showing capability of neutralizing the 40 kDa ST-MTFase activity were selected for further characterization and were used in various immunoassays as well as an immunoaffinity agent for enzyme purification.

(B) Studies of norsolorinic acid reductase (NSR): Both Mab and Pab against NSR reductase

were obtained in our laboratory. Details for the preparation and characterization of these antibodies were described in a manuscript (Publication 3) and in previous reports for this workshop. Further studies were made for the Mab produced by hybridoma cell line 10D2 during this year. The Mab produced by this clone has also been used successfully for screening the cDNA library in cloning the gene for the enzyme and in monitoring the enzyme produced in *Escherichia coli* in which the gene was expressed. We have also established an effective ELISA method for the presence the enzyme in the fungal mycelium.

(C) Characterization of antibodies against Ver-1 and Ver-A encoded protein: During our study of characterizing Pab against NSR by Western blot analysis, we found that the Pab also reacted with a 30 kDa protein species. Further characterization revealed that the 30 kDa protein actually was the protein encoded by Ver-1 gene. Consequenlty, the antiserum was purified by ammonium sulfate precipitation and an affinity column in which the Ver-1 protein extract (expressed in *E. coli*) was conjugated. Western blot analysis of fungal extracts and extracts from *E. coli* strains containing the Ver-1 and Ver 1-A genes revealed that the purified Pab reacted primarily with the 30 kDa Ver-1 protein as well as the fused 74 kDa (29 kDa + 45 kDa) protein species. These data provided strong evidence showing that the pab was indeed generated from the Ver-1 protein from the partially purfied fungal extract and that Ver-1 and Ver 1-A genes encoded the same protein species, possiby involving the conversion of versiconal hemiacetal to versicolorin A. An effective ELISA method for the enzyme was then established.

(D) Kinetics studies on the formation AFB1 by 14 fungi: With the availability of effective ELISA methods for the three enzymes discussed above, we have analyzed the kinetics of AFB1 formation by 14 fungi and examined the correlation of enzyme formation and toxin production over a period of 10 days. The AFB1 producing- *Aspergillus* used in the study were *A. flavus* strain 1273, 2111, 3.124, and *A. parasiticus* 13007, 3. 2890. The AFB1 non-producers were *A. parasiticus nor-1, A. versicolor* 147, *A. nidulan, A. oryzae* 451, 3800, *A. sojae* 6271, *Penicillium citrinum* 434 , *Fusarium* sporotrichoides T-422, and *Cochliobolus safirus.* Several conclusions can be drawn: (1) production of AFB1 is related to the presence of NSR, Ver-1 and ST-MTFase in the fungal extracts; (2) accumulation of the enzymes in the mycelium generally occurred prior to the toxin formation; (3) small amounts of NSR, Ver-1 and ST-MTFase were detected in the mycelium of some non-toxicogenic *Aspergillus* but not in other fungi; (4) The presence of NSR, Ver-1 and ST-MTFase in the non-toxic *Aspergillia* suggests that the regulator genes may play an important role in AFB1 formation.

Publications:

1. Liu, B. H., Keller, N. P., Bhatnagar, D., Cleveland, T. E. and Chu, F. S. 1993. Production and characterization of antibodies against sterigmatocystin O-methyltransferase. Food & Agric. Immun. 5:155-164.

2. Yu, J., Cary, J. W., Bhatnagar, D., Cleveland, T. E., Keller, N.P. and Chu, F. S. 1993. Cloning and characterization of a cDNA from *Aspergillus parasiticus* encoding an O-methyltransferase involved in aflatoxin biosynthesis. Appl. Environ. Microbiol. 59: 3564-3571.

3. Lee, R. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E. and Chu, F. S. 1994. Production and characterization of antibodies against norsolorinic acid reductase. Food & Agric. Immun. (In press).

POSTER

The Regulation of the *nor*-1 gene in *Aspergillus parasiticus*.

Frances Trail and John Linz. Michigan State University.

The phytopathogenic fungus, *Aspergillus parasiticus*, produces the toxic secondary metabolite aflatoxin. Coordinate expression of the *nor*-1 gene and several other genes associated with aflatoxin biosynthesis indicates that these genes are similarly regulated. Analysis of the promoter regions of these genes should provide some insight into the mechanism of this regulation. Proteins were isolated from nuclei of *A. parasiticus* grown in either aflatoxin inducing (GMS) or noninducing (PMS) medium. Mobility Shift DNA Binding assays were performed using these preparations on a 224bp region upstream of the transcript initiation site of *nor*-1. Two sites were identified that bound proteins unique to the GMS cultures. One of the sites was examined more closely by competition assays. The results indicated that the TATA box region previously identified in nucleotide sequence analysis is functionally important in binding of a protein(s) present in nuclei grown in GMS. A reporter gene fusion vector (pAPGUSN), constructed by fusing the *nor*-1 promoter to the *gus* gene, was introduced into the *nor*-1 site in the chromosome of *A. parasiticus* by a double crossover event. Aflatoxin biosynthesis and GUS activity were measured in inducing medium in a parental strain and a transformant containing pAPGUSN. The timing of the accumulation of GUS activity in the transformed strain was similar to the timing of the accumulation of aflatoxin in the parental strain. Transformants were also grown in PMS (noninducing) for 72 hrs, then shifted to GMS (inducing) to which black pepper extract had been added. Mycelial extracts from controls (GMS without pepper extract) showed high GUS activity, whereas none was detected in extracts from cultures to which black pepper extract had been added. Since the *gus* gene is under the control of the *nor*-1 promoter, inhibitors in the pepper extract have entered the cells and affected gene expression possibly at the level of transcription. Developing peanut pods were inoculated with spores from a strain of *A. parasiticus* carrying the pAPGUSN fusion gene. GUS activity was expressed in mycelia growing on the pod and testa, indicating the *nor*-1 gene is expressed. Analysis of the promoter regions of genes involved in aflatoxin biosynthesis, using the methods presented here, should provide some insight into the mechanism of regulation, a process that could lead to novel approaches to the elimination of aflatoxin from food supplies worldwide.

**GUS Reporter Assay: Detection of an Aflatoxin Inducing Component from Maize Kernels.** Mark A. Weaver and Charles P. Woloshuk, Purdue University, West Lafayette, Indiana 47907

The long term goal of this research is to determine the underlying biochemical reasons why certain seeds (maize, peanut, cotton, and pistachio) are vulnerable to aflatoxin contamination. The question that this research attempts to answer is: Are there common seed metabolites that induce or stimulate aflatoxin biosynthesis in *Aspergillus flavus*? While it is known that environmental factors lead to increased infection by *A. flavus* and aflatoxin production, there is little knowledge about what seed tissues and metabolites influence aflatoxin biosynthesis. This research project focuses on the susceptibility factors that lead to high levels of aflatoxin in maize kernels. The rationale for identifying metabolites that stimulate aflatoxin production is the importance of understanding what seed components influence secondary metabolism in *A. flavus*. With this understanding, we then can evaluate the feasibility of altering the production of these inducing metabolites in maize by classical breeding methods. It may also be posssible to develop strategies that inhibit the utilization of these inducing metabolite by the fungus.

The technical progress in molecular biology has provided new and powerful tools for studying *A. flavus* and aflatoxin biosynthesis. Several genes involved in aflatoxin biosynthesis have been isolated and characterized. It is well documented that aflatoxin production closely follows the transcription of the aflatoxin genes. Because of this linkage, it was hypothesized that an assay could be developed monitoring the transcription of the aflatoxin genes with molecular reporter gene techniques. Such an assay, would signal when the aflatoxin pathway is activated and thus could be used to identify substances that affect aflatoxin biosynthesis. Towards developing such an assay, Payne and coworkers at North Carolina State University have utilized the *E. coli*, ß-glucuronidase (GUS) gene. This reporter catalyzes the cleavage of 4-methyl-umbelliferyl-glucuronide to a product that can be easily quantified by its fluorescence and of x-gluc to a blue product that is useful in histological studies. Payne, *et al.* have cloned the GUS reporter gene to the promoter of the *ver* gene from *A. flavus*. The *ver* gene product is involved in the conversion of versicolorin A to sterigmatocystin in the aflatoxin biosynthetic pathway. The *ver* promoter was cloned in front of the GUS gene to generate the promoter-GUS construct, GAP 13-22. Subsequently a transgenic strain of *A. flavus* (656-2 GAP 13-22) was obtained that produces aflatoxin and harbors the GAP 13 construct. Payne's laboratory also has shown that in this strain, GUS expression parallels both aflatoxin production and *ver* gene transcription.

Our laboratory has developed an assay procedure for detecting substances that induce aflatoxin biosynthesis using *A. flavus* strain 656-2 GAP 12-22 and the GUS-based analysis. We have used this assay procedure to identify an inducing activity in maize kernels (Pioneer 3369A). This inducing activity was water extractable from finely ground corn kernels. When *A. flavus* NRRL 3357 was first grown on the ground kernels, higher inducing activity was recovered. These data suggest that fungal growth can facilitate the release of the inducing activity from the kernel tissues. It was also determined that the majority of the inducing activity was heat stable. We are currently pursuing research that will lead to the purification and characterization of the inducing molecule(s).

POSTER

*AFL-1* IN *ASPERGILLUS FLAVUS* AFFECTS THE EXPRESSION OF AFLATOXIN GENES. Charles P. Woloshuk and Galina L. Yousibova, Purdue University, West Lafayette, Indiana 47907.

Aflatoxins are toxic and carcinogenic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Fourteen years ago, K. E. Papa reported that strain 649 of *A. flavus* contained a dominate mutation at the *afl-1* locus resulting in the loss of aflatoxin production. By the parasexual cycle, we crossed strain 649 with the aflatoxigenic strain 86 (*afl-1*$^{+}$) to obtain diploids. These diploids did not produce aflatoxin indicating that the *afl-1* mutation is a dominate allele as described by Papa. We also compared the transcript levels of four aflatoxin genes (*nor, ver, omt* and *aflR*) in strain 649 and in the aflatoxigenic strains 86 and NRRL 3357 during the induction period of aflatoxin biosynthesis. Over a 24 hr period, no transcripts were detected in strain 649. In contrast, expression of all four genes occurred in the aflatoxigenic strains. In two diploids (649x86) that were also examined, no expression of the structural genes (*nor, ver, and omt*) was detected; however, there was expression of the regulatory gene *aflR*. These data suggest that the inhibition of aflatoxin biosynthesis associated with the *afl-1* mutation is not totally by suppression of *aflR*, but may also involve direct suppression of *nor, ver,* and *omt*.

# Structure and function of *uvm*8, a gene involved in polyketide backbone synthesis of the aflatoxin biosynthetic pathway of *Aspergillus parasiticus*.

N. Mahanti[1], D. Bhatnagar[2] and J.E. Linz[1]
[1]Michigan State University, [2] USDA ARS, New Orleans.

A novel gene associated with the aflatoxin biosynthetic pathway was cloned by genetic complementation of an *A. parasiticus* mutant strain called uvm8. The uvm8 mutant was obtained by UV mutagenesis of an *A. parasiticus* mutant strain B62 (*nia*D, *brn*-1, *nor*-1), which accumulates norsolorinic acid (NA). Metabolite conversion studies confirmed that the second block in the aflatoxin B1 (AFB1) biosynthetic pathway in uvm8 occurred prior to NA. The uvm8 is therefore a double mutant and has two blocks in the AFB1 pathway, one block at nor-1 and the other one prior to *nor*-1. The uvm8 mutant was complemented using the cosmid clones NorA and NorB enabling it to synthesize aflatoxin. By comparing the maps of cosmids NorA and NorB we narrowed down the location of *uvm*8 gene to a *Sac*1 (~8 Kb) fragment contained on cosmid NorA. When this fragment was used to transform uvm8, transformants were generated which accumulated NA. These data strongly suggested that the gene prior to *nor*-1 is localized on the ~8 kb *Sac*1 fragment. Transcript mapping of the *Sac*1 fragment suggested that the *uvm*8 transcript is 7.5 kb in size. Using a restriction endonuclease fragment derived from the *uvm*8 coding region, *uvm*8 gene was disrupted in *A. parasiticus* B62, a NA accumulating strain derived from NRRL 5862. The disrupted strain does not accumulate any detectable amounts of NA, AFB1 or other pathway intermediates demonstrating conclusively that *uvm*8 gene functions in AFB1 biosynthesis prior to NA. A portion of *uvm*8 gene has been sequenced and the deduced peptide sequence shows a high degree of similarity to the *FAS*1 gene of *Yarrowia lypolytica* including a short section of an enoyl reductase functional domain suggesting a role for *uvm*8 gene in some stage of polyketide backbone synthesis.

POSTER

# CHITINOLYTIC BACTERIA AS A SOURCE OF ANTIFUNGAL GENES TO CONTROL AFLATOXIN PRODUCING FUNGI.

R. Boyapati[1],A. L. Moyne[1], S.V. Karyala[1], P.A. Gay[1], T. E. Cleveland[2] and S. Tuzun[1], Dept of [1]Plant Pathology, Auburn Univesity, AL 36849 and[2] Southern Regional Research Center ARS. USDA, New Orleans, LA 70124.

Success of transgenic expression for the control of fungal pathogens relies upon the availability of genes encoding antifungal proteins that are active against pathogens as well as transportation of the active protein to the site of infection. Chitinases are antifungal enzymes that degrade fungal cell walls by hydrolyzing the β–1–4 linkages of chitin. Chitinolytic bacteria, as sources of antifungal genes, were tested *in vitro* for their antagonistic effects against the aflatoxin-producing fungi *Aspergillus flavus* and *Aspergilus parasiticus*. Work done in our laboratories identified an isolate of *Bacillus chitinosporus* (AU192), among 100 chitinolytic bacteria, with very high lytic activity against *Aspergillus* spp. Microscopic observations showed hyphal tip bulging and lysis as evidence of chitinolytic activity. The aim of this project is to clone and characterize chitinase genes from AU192. The strategy adopted for cloning the *Bacillus* chitinase genes, was based on the screening of a gene library for the presence of the clearing zone. DNA from AU192 was isolated and partially cleaved with Sau3AI and fragments ranging from 2-10Kb in size were purified. The DNA fragments were ligated into pUC18 and the ligation mixture was used to transform *E. coli*. A genomic library was constructed in *E. coli* and screened for presence of clearing zone around colonies on a chitin containing media. The ultimate goal of our project is to express a bacterium-derived antifungal chitinase gene constitutively in transgenic peanut and to reduce aflatoxin contamination.

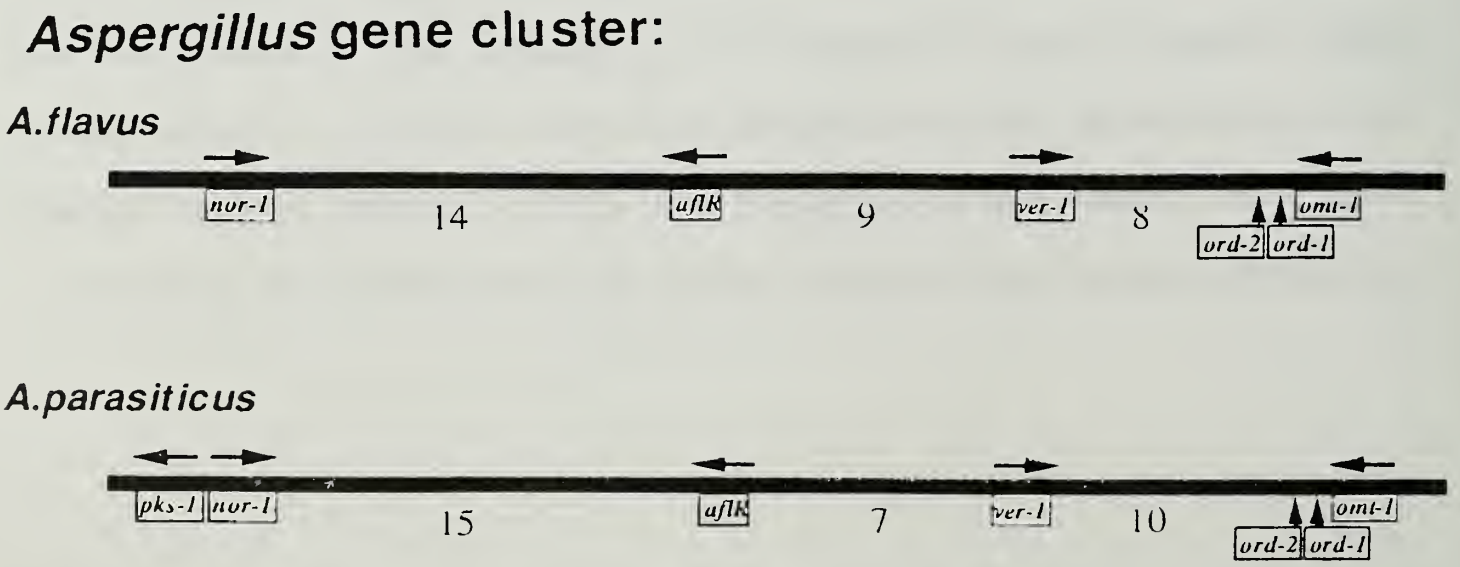
POSTER

**Molecular regulation of aflatoxin biosynthesis: Comparative mapping of aflatoxin pathway gene clusters in aflatoxigenic fungi *A. flavus* and *A. parasiticus***

J. Yu[1], P.-K. Chang[2], D. Bhatnagar[1], T. E. Cleveland[1], J. W. Cary[1], J. E. Linz[3], G. A. Payne[4] and J. W. Bennett[2].

[1]USDA/ARS/SRRC, New Orleans, LA; [2]Tulane University, New Orleans, LA; [3]Michigan State University, E. Lansing, MI; and [4]North Carolina State University, Raleigh, NC.

Aflatoxins are secondary metabolites produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* that infect corn, cotton, peanut and tree nuts. These compounds are toxic and carcinogenic to animals and present a potential health hazard to human beings. In order to devise strategies for reducing and eliminating aflatoxin contamination from food and feed, extensive biochemical and genetic studies are being conducted to better understand the molecular mechanisms which regulate aflatoxin biosynthesis. Aflatoxins are synthesized by condensation of acetate units; their biosynthesis is estimated to involve at least sixteen different enzymes. Genes encoding four of these enzymes including *pks-1, nor-1, ver-1* and *omt-1*, as well as a gene *aflR*, which regulates transcription of the pathway genes have been cloned in our laboratories. We report here the comparative organization and arrangement of the aflatoxin pathway genes and a regulatory gene on the *Aspergillus parasiticus* and *A. flavus* chromosomes. By determining overlapping regions of the inserts in cosmid and lambda clones, the aflatoxin pathway genes were located within a 60 kb DNA fragment in the order of *pks-1, nor-1, aflR, ver-1* and *omt-1* in both *A. parasiticus* and *A. flavus*. This order is coincident with the order of the activities encoded by these genes in the biosynthetic pathway, with the exception of the regulatory gene, *aflR*, which is located between the *nor-1* and *ver-1* genes. The distances (in kilobase pairs) between these genes have been determined. Immediately downstream from the *omt-1* gene transcription, two additional transcripts (1.4 kb and 1.5 kb, respectively) were identified which may also be involved in aflatoxin biosynthesis. The identities of these two genes are being investigated.



POSTER

**Fingerprints in the *aflR* gene and its homologs among members of *Aspergillus* Section *Flavi***

P-K. Chang[1], D. Bhatnagar[2], M. A. Klich[2], T. E. Cleveland[2] and J. W. Bennett[1]

[1]Tulane University, New Orleans, LA; [2]USDA/ARS/SRRC, New Orleans, LA

The *Aspergillus parasiticus aflR* gene, a gene potentially involved in the regulation of aflatoxin biosynthesis, encodes a putative zinc finger DNA-binding protein. Polymerase chain reaction (PCR) and sequencing were used to examine the presence of *aflR* homologs in other members of the *Aspergillus* Section *Flavi*. Predicted amino acid sequences indicated that the same zinc finger domain, CTSCASSKVRCTKEKPACARCIERGLAC, was present in all the *A. sojae, A. flavus* and *A. parasiticus* and in some of *A. oryzae* isolates examined. Unique base substitutions and a specific base deletion were found in the 5' untranslated and zinc finger region, which constituted distinct fingerprints. *A. oryzae and A. flavus* had the T-G-A-A-X-C fingerprint whereas *A. parasiticus* and *A. sojae* had the C-C-C-C-C-T fingerprint at the corresponding positions. Specific nucleotides at -90 (C or T) and -132 (G or A) further distinguished *A. flavus* from *A. oryzae* and *A. parasiticus* from *A. sojae* respectively. *A. sojae* ATCC 9362, previously determined as *A. oryzae* NRRL 1988, was determined to be *A. sojae* based on the presence of the characteristic fingerprint, A-C-C-C-C-C-C-T. Other members of *Aspergillus* Section *Flavi*, such as *A. nomius* and *A. tamarii*, and some isolates of *A. oryzae* appeared to have low DNA similarity to the *A. parasiticus aflR* gene since few or no PCR products were obtained when DNA from these strains was used.

POSTER

# HOMOLOGY OF GENES IN THE AFLATOXIN BIOSYNTHETIC PATHWAY OF *ASPERGILLUS PARASITICUS* TO DNA OF OTHER FUNGAL TAXA

Klich, M.A.[1], Yu, J.J.[1], Chang, P-K.[2], Mullaney, E.J.[1], Bhatnagar, D.[1], and Cleveland, T.E.[1] [1]U. S. Department of Agriculture, A.R.S., Southern Regional Research Center, New Orleans LA USA. [2] Department of Cell and Molecular Biology, Tulane University, New Orleans LA USA.

Several genes involved in the biosynthesis of aflatoxin have been cloned. In this study, we examined homology of two of these genes, *omt*-1 and *aflR* to DNA of aflatoxin-producing isolates of *A. flavus* and *A. parasiticus*, and non-toxigenic strains of these and other related species. The *omt*-1 gene is involved in the formation of o-methylsterigmatocystin, the penultimate step in biosynthesis of aflatoxin $B_1$. The *aflR* gene is a regulatory gene which apparently governs several steps in the aflatoxin biosynthetic pathway. DNA from a number of *A. flavus, A. parasiticus, A. oryzae*, and *A. sojae* isolates as well as several other related taxa was digested with the restriction enzyme *Eco*RI and probed with the genes. All *A. flavus, A. parasiticus* and *A. sojae* isolates examined had regions of homology with both genes. *Aspergillus oryzae* and *A. tamarii* had no visible regions of homology to the *aflR* gene. The one putative *A. oryzae* isolate that did have homology to the *aflR* gene, was found to be a misidentified *A. flavus*.

**Analysis of the Sterigmatocystin Gene Cluster of *Aspergillus nidulans*.**

Daren Brown[1], Hemant Kelkar[2], Robert Butchko[1], Clint Nesbitt[1], Mary Fernandes[2], Suzanne Segner[1], Deepak Bhatnagar[3], Nancy Keller[1], Tom Adams[2]

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**SUMMARY**

*Aspergillus nidulans* produces the polyketide sterigmatocystin (ST), a carcinogenic secondary metabolite. ST is the next-to-last precursor in the aflatoxin (AF) biosynthetic pathway found in the related fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Our prior studies have described a gene, *verA*, encoding a keto-reductase which is necessary for ST biosynthesis in *A. nidulans*. The *verA* gene product bears 85% amino acid identity to the *A. parasiticus ver-1* gene product. We have found that *verA* is flanked on either side with at least 20 transcripts which are co-regulated with *verA* transcription. We suggest that (i) these 20+ transcripts constitute the ST gene cluster in *A. nidulans* and that (ii) both enzymatic and regulatory gene function is conserved between the ST gene cluster of *A. nidulans* and the AF gene cluster in *A. flavus*/*A. parasiticus*.

The ST cluster (approximately 60 kb) is located within three overlapping cosmids on chromosome 4 in *A. nidulans*. Sequence comparison (of regions corresponding to the transcripts) to known genes in the databases have identified a number of activities consistent with the biosynthesis of sterigmatocystin including a polyketide synthase (PKS), a fatty acid synthase (both FAS a and ß), 6 monooxygenases, 1 esterase, 2 dehydrogenases, 1 methyltransferase, and 2 potential regulatory genes. The latter group includes the *A. nidulans* homolog (*anaflR*) of the *A. flavus*/*A. parasiticus* regulatory gene, *aflR* (1, 2, and 5). Vectors designed to disrupt #17 (ORF1- g subunit of elongation factor 1), #9 (FAS a) and #16 (ORF2- cytochrome P450 monooxygenase) have been constructed. Disruption of #16 (designated *verB*) results in a block of the ST pathway at versicolorin A.

We have also shown that regulatory functions are conserved between *A. flavus, A. parasiticus* and *A. nidulans*. The *A. flavus* AF regulatory gene, *aflR* (a putative transcriptional factor; 1, 5), fused to the *A. nidulans niiA* inducible promoter functions to regulate ST genes in *A. nidulans*. Northern analysis of an *A. nidulans* transformant containing the *niiA::aflR* construct has demonstrated that induction of *niiA::aflR* results in concomitant activation of *verA*. We have also identified the *A. nidulans aflR* homolog, *anaflR* by designing a primer to the conserved zinc finger domain of *aflR* and using this primer to locate and sequence *anaflR*. We find that *anaflR* (#4) lies between the PKS (#1) and the FAS (#9 and #10) in the ST gene cluster.

POSTER

## The Sterigmatocystin Gene Cluster in *Aspergillus nidulans*

Thomas J. Leonard and Jaehysuk Yu

We employed a large induced deletion mutant, which did not produce sterigmatocystin (STC) nor any pathway intermediates, to study the gene cluster associated with STC biosynthesis. In initial experiments we screened an *Aspergillus nidulans* cosmid library with a nor-1 cDNA clone from *A. flavus* (provided by G. Payne) and identified a cosmid, pL11C09, which overlapped (2.0 kb) with a ver A containing cosmid, pL24B03. The chromosomal region spanning the deletion included all of cosmid pL11C09 and ca. 5 kb of pL24B03, terminating in the verA gene. To determine the other end of the deletion we carried out chromosome walking and identified several cosmids which extended beyond cosmid pL11C09. Analysis showed the deletion extended ca. 10 kb beyond cosmid pL11C09 indicating the size of the deletion was ca. 55 kb. Using a restriction map of the corresponding wild type region, probes were prepared for northern analysis and a transcription map was established for the entire deletion region. Twenty transcripts including the STC polyketide synthase (PKS), *nor-1*, *verA* and an *aflR* homolog, *anaflR* were identified . We carried out gene disruption with PKS and anaflR. When PKS was disrupted, no STC was produced but all of the pathway genes including anaflR produced transcripts under STC promoting conditions. When anaflR was disrupted, no STC was produced nor were any STC gene-related transcripts detectable. Thus anaflR, like its counterparts in *A. flavus* and *A. parasiticus*, serves as an activating regulatory element. The anaflR transcript is produced at a low basal level until stimulated by STC promoting conditions to produce high levels of transcript, which activated the related STC gene cluster. Both the PKS and anaflR genes have been sequenced. The PKS gene sequence showed highest homology to a pigment-associated PKS encoded by the *wA* gene of A. *nidulans*, and to various PKS's produced by Streptomyces. The anaflR sequence showed a serine rich region, which may be associated with phosphorylation and the activation process, a $C_2C_6$ Zn finger, which is commonly found among DNA binding proteins, and an activation domain. We proposed several models on how anaflR might act in activating the STC gene cluster and both involving a signal transduction mechanism.

**Panel Discussion Summary: Aflatoxin Biosynthesis as a Critical Tool in Aflatoxin Elimination**

**Chair: D. Bhatnagar**

**Panel: F-S. Chu, N. P. Keller, T. Leonard, J. E. Linz, G. A. Payne, and C. Woloshuk**

The following approaches that have application to all aflatoxin-susceptible crops are being examined to try to exclude toxigenic fungi from their environmental niches or to at least reduce aflatoxin biosynthesis in case host tissues are colonized by the fungi:

1. **Agronomic Practices. Cultural practices are designed and evaluated to reduce aflatoxin contamination.**
2. **Fungal Ecology: A biocompetitive approach. Replacing aflatoxigenic strains with nonaflatoxigenic strains in the field.**
3. **A Host-Plant Resistance Approach: a) Genetic Engineering of Crops. Incorporate antifungal genes expressed in the specific plant tissues, e.g., seed tissues contaminated by aflatoxigenic strains, or b) Plant Breeding: Assess germplasm for development of varieties resistant to fungal invasion or aflatoxin production.**

The above mentioned approaches for elimination of preharvest aflatoxin contamination could, in some instances, involve specific requirements depending on crop or environmental conditions. It means that each individual host system must be studied in detail to understand the particular permutations associated with each and how best to exploit each of these systems. For example, the mode of entry of the fungus may be different in cotton than in peanuts or tree nuts. The fact that waxes on corn kernel surfaces may be involved in resistance in that crop may be not related at all to peanuts. Also, the fungal strains present in Arizona may have different survival characteristics than those found in Iowa.

The aflatoxin biosynthetic pathway is a common feature in all aflatoxin contamination problems and can be more readily understood. Successful and efficient implementation of the molecular strategies for aflatoxin control could involve understanding the molecular genetics of aflatoxin biosynthesis. A detailed description of the potential contributions of molecular biology to aflatoxin elimination are detailed in an article to be published in an upcoming issue (March, 1995) of INFORM, a popular publication of the American Oil Chemists' Society.

## Recent Accomplishments in Molecular Biology of Aflatoxin Biosynthesis

Several labs are involved in studying the molecular regulation of aflatoxin biosynthesis. Over the years (since 1990), funding for research in aflatoxin biosynthesis has averaged about 13-15% of the total funding provided through the Competitive Award Program authorized by Congressional appropriations (Table 1). A comprehensive and cooperative research effort among the scientists, listed as part of the panel, has resulted in several discoveries in the last few years. The chemistry, biochemistry, and genetics of the biosynthetic pathway have been characterized in details (Figure 1). The regulation of aflatoxin biosynthesis has been elucidated and the organization on the fungal chromosome of the genes involved in toxin synthesis has been understood.

## Benefits of Cloned Biosynthetic Genes in Aflatoxin Elimination

The complete characterization of aflatoxin genes and their regulation will be extremely beneficial to other projects seeking to develop non-aflatoxigenic biocompetitive fungi or to monitor crop resistance to fungal growth and aflatoxin formation. These promising areas of research are discussed below and are summarized in a schematic (Figure 2).

**(a) Producing a characterized biocontrol strain** Native strains of *A. flavus* already are available which produce little or no aflatoxin and these were effective biocompetitive agents in small scale field trials. However, we now know that some of these strains contain functional traits/genes that govern the aflatoxin pathway from the earliest known precursor (norsolorinic acid) to aflatoxin. The mechanism behind lack of aflatoxin synthesis in these strains is unknown, but it is generally thought that a regulatory element or even an earlier biosynthetic step is missing. Thus, for attaining complete confidence in the ability of a biocontrol strain to not produce toxins, it would be desirable to selectively remove genes governing aflatoxin production without reducing the biocompetitive value of the fungus. Also, more fundamental knowledge about the lack of ability to make toxin would be desirable in the regulatory approval process for use of biocompetitive *A. flavus* strains. In the area of quality control, products involving engineered strains could be easily verified by their "signature," a lack of certain gene coding regions.

In this effort, genes in the aflatoxin pathway gene cluster have been selectively inactivated through gene disruption techniques. The effects of these gene inactivation on aflatoxin biosynthesis, ability of disrupted strain to colonize plants, biocompetitive ability and other phenotypic effects of gene disruption are currently being monitored. Since genes regulating aflatoxin biosynthesis have been identified, the effects of signals or compounds from plants or environment that either trigger or inhibit aflatoxin biosynthesis now can be monitored. Evidence is accumulating that compounds exist in crop plants that inhibit aflatoxin biosynthesis and a mechanism may exist in plants (so called resistant varieties that do not accumulate aflatoxin) to be able to turn off aflatoxin biosynthesis. These observations could shed light on the ecological significance to the fungus of the biosynthetic pathway.

**(b) Monitoring host-plant resistance** An immediate use of aflatoxin gene cloning has been the development of a gene-specific assay to screen plant genotypes. Such an assay is based on the ability of plant extracts to inhibit or induce promoter activity of one of the pathway genes. Several strategies to monitor such an activity have been discussed. A protocol, developed by Drs. Payne and Woloshuk, is being tested at their labs as well as at the USDA/SRRC lab, since this appears to be the most practical assay procedure for determining when and where the aflatoxin production is turned on *in vivo*. The protocol developed measures promoter activity of pathway genes by fusing the promoter to a reporter gene such as GUS gene (for β-glucuronidase, an enzyme that hydrolyzes glucuronides to produce colored or fluorescent products). The expression of GUS activity during aflatoxin elaboration has been measured by the production of an easily visualized compound in corn kernels. The advantages of such an assay are: 1) it is easier and safer than aflatoxin analyses because no elaborate solvent separation techniques involved, 2) it is more sensitive and less variable than routine chemical analysis because it directly measures a gene in the pathway, and 3) it will allow the identification of compounds

that block the gene expression in the early, middle or late steps in the pathway (since genes have now been cloned from various stages in the pathway).

One goal of these research programs is to assist plant breeders in developing plants resistant to aflatoxin contamination. Extensive efforts in this area by some of the best plant breeders have failed to develop resistant lines. At this point, plant breeders have no specific markers to aid in selection of resistant genotypes and no compounds have been identified that can be easily incorporated into desirable genotypes. Aflatoxin biosynthetic genes are the ultimate markers which could be used in breeding for resistance as "Tester Strains." These genes will be the analytical tools for measuring "resistance" or "susceptibility" of plant tissue. In a screening program, the aflatoxin genes will be the "micro-probes" which will be transported by the invading fungus (*A. flavus* or *A. parasiticus*) into crop/seed tissues (aleurone, embryo, endosperm, etc.) to give a "reading" on the location and quantity of chemical inhibitors. Efforts are being focused in our labs to provide this technology which has been developed and can be easily implemented on a practical basis. Thus, aflatoxin genes discovered recently in the cooperating laboratories on a gene cluster will be used as selectable markers in a plant breeding approach by linking them to easily assayed reporter genes such as the GUS gene; tissues can be assayed visually for levels of blue color or fluorescence (depending upon the substrate used) due to GUS. High resistance to aflatoxin contamination would be correlated with low GUS activity. Plant traits inducing aflatoxin would lead to high GUS activity.

The above-mentioned gene approach is noted for its simplicity and could be utilized in most plant breeding laboratories. One only needs the fungal strain carrying the aflatoxin gene-GUS gene construct (which we would engineer) and the substrate for GUS. Selection for resistance to aflatoxin contamination might not require time consuming field trials since preliminary varietal trials might be accomlished using single kernel assays in the laboratory using the GUS assay. Preliminary results using single corn kernels have correlated well with resistance to aflatoxin in field tests carried out by plant breeders. The procedure is pragmatic and depends only upon the fungus' natural ability to infect and to transport the "gene expression probe" into plant tissue sites where the aflatoxin inducer/inhibitor chemicals reside. These gene probes could, therefore, detect and quantitate important resistance traits in crop tissues regardless of the chemical nature of the trait or its tissue location. Fungal growth associated genes linked to the GUS reporter gene already have been utilized to visualize *A. flavus* invasion in individual kernel tissues (SRRC and University cooperators). Therefore, this method has great potential for detecting both fungal growth and aflatoxin expression and for locating and differentiating unknown traits, which perhaps vary by tissue site. A longer-term technology which could be derived from the identification of resistant varieties by this method is that plant inducer/inhibitor chemicals (and subsequently genes governing their synthesis) can be identified using the gene probe method. These genes probes could be used in genetic engineering of any crop subject to aflatoxin contamination.

If and when genetically engineered cotton, corn, and peanut plants containing genes that impede fungal growth are obtained, an assay similar to the one described above likely will be utilized to measure the resistance against not only growth but also aflatoxin biosynthesis. Even though the transformation of plants/seeds with antifungal genes may greatly reduce fungal growth in certain seed/tissue compartments, the remainder of fungal growth in other tissues may still produce

significant aflatoxin, and in fact be enhanced due to chemical stress on the fungus. Thus, it might appear initially that little benefit occurred from incorporation of the antifungal gene. Therefore, the monitoring of specific tissue/seed compartments for aflatoxin elaboration with easily visualized reporter genes specifically linked to aflatoxin production will be a more definitive measure of resistance and the exact location of resistance expression in tissues of genetically engineered crop plants. Tje sa,e assau (GUS constructs) could be used for monitoring fungal growth or aflatoxin production under various agronomic conditions.

### (c) Other Long-Term Basic Research Benefits

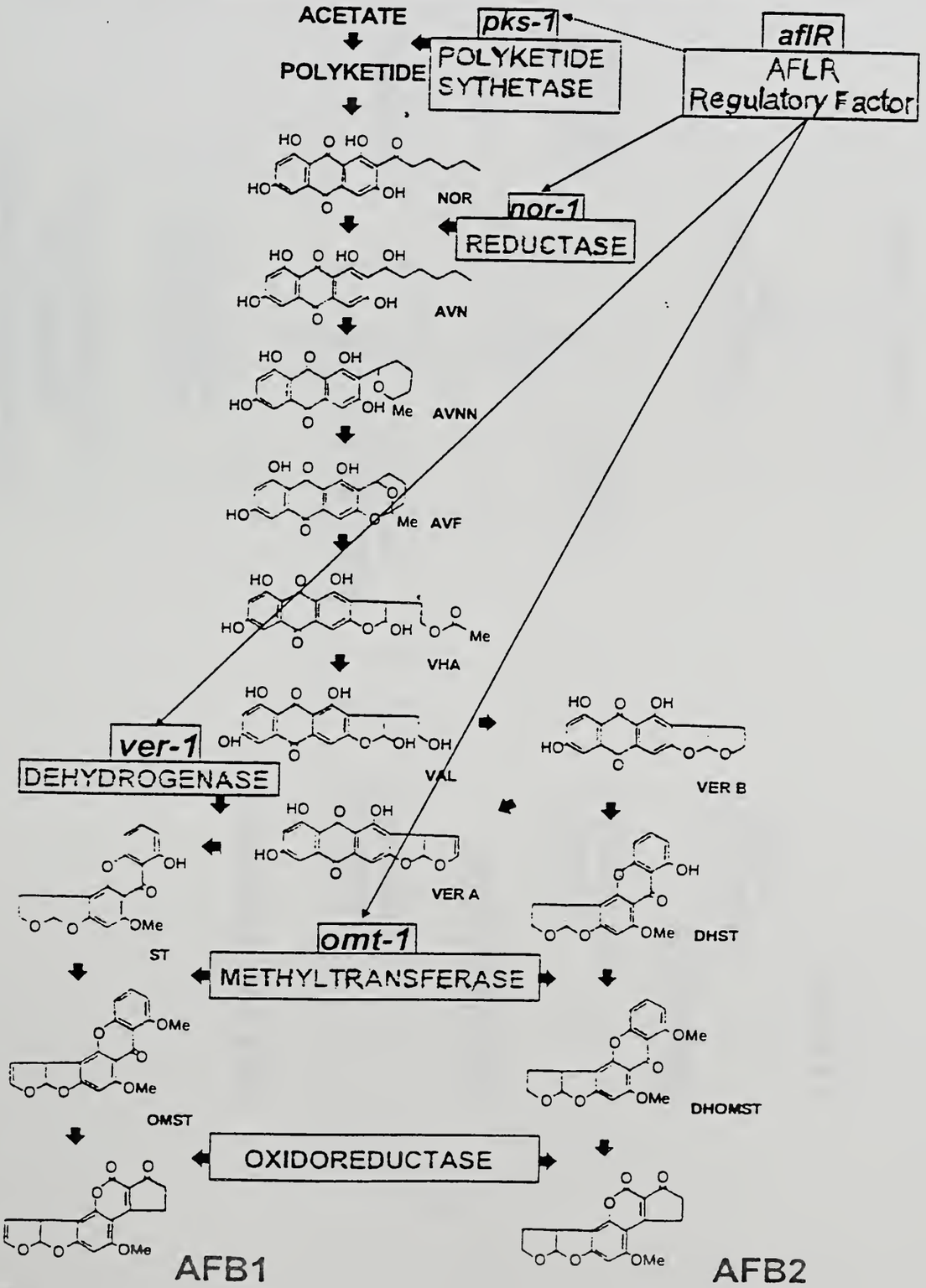
Until now, no obvious role in fungal growth and survival has been assigned to the genes controlling aflatoxin formation. There is increasing evidence that genes involved in aflatoxin biosynthesis may play a role in survival of the fungus because sclerotia formation and spore pigmentation seem to be regulated in a manner similar to aflatoxin synthesis. Some specific role must exist since we now know from molecular studies of the presence of a relatively large aflatoxin pathway gene cluster. This is a significant genetic complement for such a primitive organism with a specific function of carrying out an elaborate and complicated set of oxidation-reduction reactions to synthesize aflatoxin. We also know that the "master" regulatory gene we discovered on the same gene cluster (and others that we may find) not only governs aflatoxin formation but also influences development of sclerotia which are overwintering structures in *A. flavus* and *A. parasiticus*. Also, one of the aflatoxin pathway genes is very similar to a melanin biosynthesis gene in another fungus suggesting a possible role for this pathway gene in formation of melanin, a pigment sometimes essential to fungal survival. Therefore, it is with the understanding of the genetics governing aflatoxin biosynthesis that we will be able to learn whether this process is required for the survival of the fungus in the environment. In this effort, the large collection of non-producing *A. flavus* native strains could provide essential clues. Once we have this answer, we can develop more targeted methods to modulate that specific genetic complement at will to control aflatoxin elaboration by eliminating fungus survival itself.

# Funding for Biosynthesis Research

Table 1

| Proposal Funded | 1990 | 1991 | 1992 | 1993 | 1994 |
|---|---|---|---|---|---|
| Payne | $58,051.00 | $54,633.00 | $35,000.00 | $38,748.00 | $38,500.00 |
| Chu | | $34,182.00 | $29,140.00 | $30,000.00 | $20,000.00 |
| Linz | | | $17,360.00 | $35,000.00 | $35,000.00 |
| Leonard | | | $20,000.00 | | |
| Gendloff / Smalley | | | $25,000.00 | | |
| Keller | | | | | |
| Woloshuk | | | | | |
| Hsieh | | | | | |
| Funding (Biosynthesis) | $58,051.00 | $88,815.00 | $126,500.00 | $103,748.00 | $93,500.00 |
| Total Funding | $331,964.00 | $692,750.00 | $802,750.00 | $802,750.00 | $790,709.00 |
| % of Total | 17.50% | 12.80% | 15.80% | 12.90% | 11.80% |

Figure 1



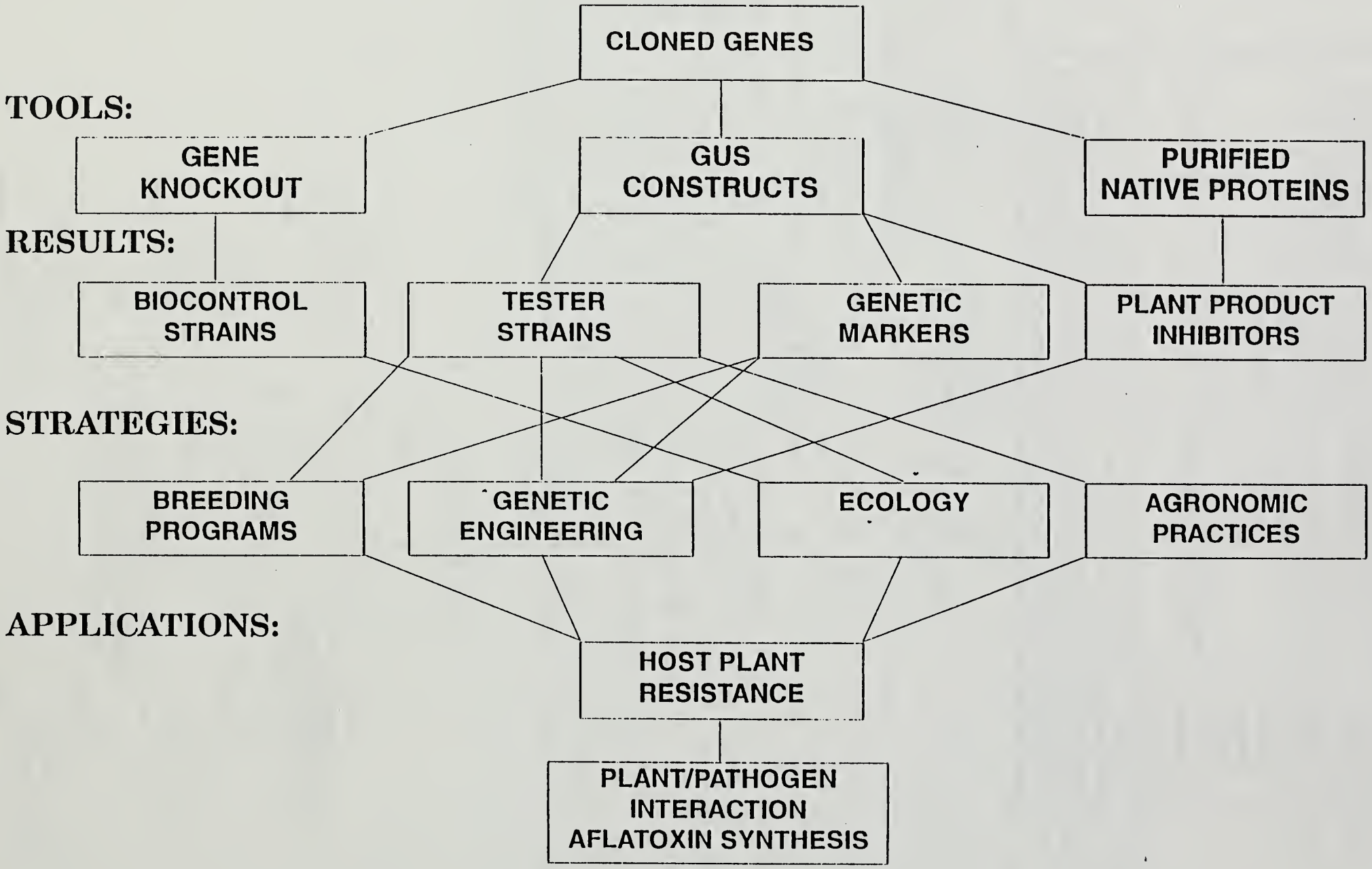

CLONED GENES
TOOLS:
GENE KNOCKOUT
GUS CONSTRUCTS
PURIFIED NATIVE PROTEINS
RESULTS:
BIOCONTROL STRAINS
TESTER STRAINS
GENETIC MARKERS
PLANT PRODUCT INHIBITORS
STRATEGIES:
BREEDING PROGRAMS
GENETIC ENGINEERING
ECOLOGY
AGRONOMIC PRACTICES
APPLICATIONS:
HOST PLANT RESISTANCE
PLANT/PATHOGEN INTERACTION AFLATOXIN SYNTHESIS

Figure 2

# MICROBIAL ECOLOGY

# Impact of Strain Dispersal on Aflatoxin Contamination in Field Plots and Commercial Crops: Etiology and Potential Amelioration of Aflatoxin Contamination.

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The complexity of dispersal of *Aspergillus flavus* strains within field plots and in the commercial crop influences the incidence and severity of aflatoxin contamination. In field experiments in the Yuma Valley of Arizona, atoxigenic strains of *A. flavus* readily disperse from application points in field plots to untreated areas. In one experiment three atoxigenic strains were applied to the same area (20 $cm^2$). All applied strains displaced other strains resident in the field during infection of developing bolls. Applied strains dispersed points in all directions and displaced other strains even 15 meters from the application area. Displacement was independent of the percent of developing cotton bolls infected by *A. flavus* and the percent infected cotton (as measured by incidence of fluorescent locules) was not increased by strain application. Strains differed in relative success in dispersing and infecting developing bolls. In sites distant from the application area, individual applied strains tended to dominate. This may suggest early strain establishment plays a vital role in dictating the composition of strains associated with the crop at maturity.

Vegetative compatibility analyses performed on four fungal isolates per infected locule indicated that cotton boll infections are typically caused by multiple *A. flavus* strains. Multiple infections were even commonly detected in individual seeds either collected from bolls deliberately inoculated by multiple strains, during greenhouse studies, or collected from the commercial crop. Thus, strain ability to reduce contamination of developing bolls during co-infection with toxigenic strains may be an important determinant of atoxigenic strain efficacy in preventing contamination in the commercial crop.

Analysis of both infecting fungal strains and aflatoxin content of infected seed indicates that strain dispersal and the complexity of infection greatly influence the extent of contamination both in test plots and in the untreated commercial crop. In a study of seed with bright-green-yellow-fluorescence collected from cotton gins in western Arizona, the highly toxigenic S strain of *A. flavus* caused more contamination of the commercial crop than the "typical" or L strain of *A. flavus*. L strain isolates caused less than 15% of the detected contamination in each of the three years during which the study was performed. Co-infections with L strain isolates resulted in lower average aflatoxin levels than infection by S strain isolates alone. This demonstrates strains with lower aflatoxin producing potential reducing the quantity of contamination caused by highly toxigenic strains in an untreated commercial crop. This is analogous to what occurs in greenhouse tests when toxigenic and atoxigenic strains are co-inoculated into developing bolls and the resulting toxin content of the mature seed is less than in bolls inoculated with the toxigenic strain alone. Greenhouse and laboratory tests indicate that these reductions in toxin content in co-infected seed are probably attributable to competition for both infection sites and nutrients.

**7 th ANNUAL AFLATOXIN ELIMINATION WORKSHOP**

*ST. LOUIS, MO. OCTOBER 23-25, 1994*

***Relationships of inoculum sources of Aspergillus flavus to control by disease resistance and management.***

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Three resistant and two susceptible hybrids, as determined by Dr. D. White, University of Illinois, were exposed to natural inoculum of *Aspergillus flavus* from waste corn obtained from a bin site in Iowa and applied to field plots just before silking at 0, 1, 5 and 10 kg/plot. Results showed that a good relationship existed between waste corn amounts and spore discharges. Husk infection was similar across hybrids, but both silk and kernel infection were lower for the resistant compared to the susceptible hybrids. Results of kernel infection in this experiment also were in complete agreement with those obtained in an artificial inoculation experiment at the same location, thus indicating that the waste corn method is an effective natural resistance screen.

An epidemiological model, proposed in 1993, suggested that a threshold value of the *A. flavus* population in July may be related to massive increases in population fungus that occur under hot, dry conditions in epidemic years. Surveys across a range of sites in Iowa confirmed the consistency of an increase in the soilborne population of *A. flavus* in field soils in July. The mechanism for this increase was studied by exposing field soil to a range of temperature and moisture conditions on a thermogradient plate and periodically measuring soilborne populations of *A. flavus*. Results showed that soil temperatures in the range 30-39 C greatly increased soil populations of *A. flavus* within 1 week of incubation.

Evidence also was obtained to explain the increase in population on corn plants that occurs under hot, dry conditions. Single plants of resistant and susceptible hybrids were grown in pots in a greenhouse until just before silking. They were transferred to growth chambers maintained at either 20 C or 30 C. One set of pots of each hybrid at each temperature had waste corn, naturally infested with *A. flavus*, placed on the soil surface and another set was left untreated. *A. flavus* infection of husks, silks and kernels was much higher for plants held at the 30 C than at 20 C. Leaf infection was similar for resistant and susceptible hybrids, but silk and kernel infection as well as aflatoxin contamination were all less for resistant compared to susceptible hybrids.

With the addition of this new data, the model for this disease now shows linkages between soilborne inoculum, which is the only realistic source of massive inoculum disease necessary for an epidemic, and aflatoxin contamination of resistant and susceptible hybrids. The model still requires further information to realize its potential as an effective management system for control of aflatoxin contamination of corn. This includes: the determination of relationships between cultural practices, early-season soilborne populations of *A. flavus*, and soil temperatures; experimental evidence that conidia of *A. flavus* are directly released into the air from soil; the establishment of an economic threshold for the soilborne population necessary for epidemics as they relate to susceptibility of hybrids; and clarification of the role of silks as a resistance factor in resistant and susceptible hybrids.

POSTER

# dsRNA in *Aspergillus flavus*: Variability Among Infections and Lack of Evidence for Transfer Among Isolates

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Double-stranded (ds) RNA molecules are widespread in plant pathogenic fungi. Phenotypic traits such as hypovirulence of *Cryphonectria parasitica*, killer strains of *Saccharomyces cerevisiae*, and toxin production by *Ustilago maydis*, are directly associated with infection of fungi by dsRNA. In addition, there is a published report suggesting that dsRNA infection is inversely correlated to the ability of certain *Aspergillus flavus* isolates to produce aflatoxins. These observations lead to questions on distribution of dsRNA within communities of *Aspergillus section flavi* and the relationship of dsRNA to aflatoxin production and phenotypic variability.

Ninety-six isolates in the *A. flavus* group were analyzed for dsRNA. These included both culture collection isolates and recent field accessions. Phenol extraction was used to extract nucleic acids from lyophilized, liquid nitrogen ground, mycelium from broth cultures. Two cycles of cellulose (CF-11) column chromatography were performed to purify the dsRNA fraction. The dsRNA was then precipitated, resuspended in STE buffer, and visualized on ethidium bromide stained 1% agarose gels.

The stability of dsRNA infection was examined. Attempts to cure 6 isolates of dsRNA by serial single-conidium transfer, selection of nitrogen metabolism mutants (nit), and cycloheximide treatment met with variable results since partial, total and no curing was observed.

Among all isolates tested, 10 (10.4%) contained at least one molecule of dsRNA. Isolate NRRL 5565, previously reported to be infected with 3 dsRNA molecules (each ~3 Kb in size), contained a single dsRNA molecule ~14 Kb in size. Molecular size estimates of dsRNA molecules ranged from 0.4 Kb to > 10.0 Kb based on electrophoretic migration of a dsDNA ladder in a 1% agarose gel, and no two isolates contained identical dsRNA electrophoretic profiles. These results suggest dsRNA genetic elements are not easily spread among isolates.

Isolates differed in tendency to retain infection by dsRNA molecules. Smaller dsRNA molecules were more frequently eliminated from isolates infected with complex mixtures of dsRNA elements. Complete elimination of dsRNA from fungal isolates infected by single dsRNA molecules occurred more frequently than complete elimination from isolates infected with multiple dsRNA molecules.

Overall, no one method of eliminating dsRNA infection was more effective than another. It appears that the size of dsRNA molecules and the complexity of the infection has more influence on elimination of dsRNA than does the elimination method.

No relationship between dsRNA infection and atoxigenicity was observed. Aflatoxin producing isolates of *A. flavus* (both S strain and L strain isolates) produced similar levels of aflatoxin both before and after curing. The atoxigenic strain (NRRL 5565) produced no aflatoxins both before and after curing. Similarly, total or partial curing of dsRNA in the *A. tamarii* and *A. nomius* isolates had no effect on aflatoxin production.

# Evolution and Adaptation Among Aflatoxin Producing Fungi

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Populations of *A. flavus* group fungi are highly diverse. This diversity is reflected in aflatoxin producing ability, vegetative compatibility groups, and morphology . However, relationships among the various *A. flavus* group members are not clear. Several molecular techniques have been applied to studying these relationships. *A. flavus* clearly has considerable morphological variability and sclerotial type can be used to separate the L and S strains of *A. flavus*. However, although polymorphisms in RAPD profile and esterase isozymes support this division, variation in the taka-amylase A gene was not sufficient to separate the two strains. The taka-amylase technique distinguished S strain isolates which produced only B aflatoxins from S strain isolates which produced both B and G aflatoxins. Cladistic analysis suggests that S strain isolates that produce both B and G aflatoxins may be ancestral to both *A. parasiticus* and *A. flavus*. Thus, if all S strain isolates are morphologically assignable to the species *A. flavus*, as suggested by previous workers, the current studies support reduction of *A. parasiticus* to variety status as suggested by Kurtzman and colleagues in Peoria. This does not alter the fact that isolates currently classified as *A. parasiticus* are readily distinguishable by clear morphological, physiological, and, as demonstrated by the current studies, genetic criteria.

The cladistic analysis suggests a sequence through which phenotypes may have evolved. In these analyses either *A. nidulans* or *A. tamarii* was designated the outgroup. Derivation of *A. flavus* and *A. parasiticus* from isolates which produce both sclerotia typical of the S strain of *A. flavus* and G aflatoxins is suggested by cladistic analysis regardless of whether *A. nomius or A. tamarii* are assigned outgroup status. The ancestral type leading to both S strain sclerotia and the large ovoid sclerotia of *A. nomius* is not clear. However, the cladistic analysis does suggest certain isolates that produce relatively large sclerotia (>300 μm average diameter), (i.e. certain *A. parasiticus* isolates and isolates belonging to the L strain of *A. flavus*) may be derived from isolates that produce small S strain sclerotia. Similarly, certain isolates producing echinulate spores (*i.e. A. parasiticus* isolates) may be derived from isolates which produce smooth walled conidia (S strain isolates), and not directly from *A. tamarii* isolates which produce conidia with very rough, thickened walls. Also, strains which produce only B aflatoxins are apparently derived from strains which produce both B and G aflatoxins. This course of speciation suggests a series of adaptations that may explain the ability to certain *A. flavus* group fungi to parasitize distantly related animal and plant hosts and utilize diverse substrates. Identifying the path of adaptation and the role adaptations play in niche occupation may eventually permit development of techniques to avoid problems caused by these fungi. Such information would undoubtedly support efforts to develop techniques to prevent aflatoxin contamination by altering *A. flavus* group populations to be less toxigenic.

The use of PCR to characterize and determine relationships among fungal strains is a relatively recent development. By altering the target of amplification, the ability of PCR techniques to resolve different fungal taxa can be altered. The ability of the taka-amylase gene technique described here to distinguish subgroups within the *A. flavus* group demonstrates the potential benefit of investigating variability within nontraditional amplification.

# Sex and Isozymes: Identification of *Aspergillus flavus* Clonal Lineages

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Isolates of *Aspergillus flavus* vary widely in many morphological and physiological traits of both economic and ecological importance. To address questions about communities composed of fungi in *Aspergillus* section *flavi*, rapid techniques to identify and distinguish community constituents are needed. Esterase isozymes have potential application in this context.

Total proteins were extracted from sixty-five isolates of several *Aspergillus* species. These included representative isolates of *A. flavus* (L and S strains), *A. oryzae*, *A. parasiticus*, *A. sojae*, *A. nomius*, *A. tamarii*, *A. nidulans*, and 28 isolates of the L strain of *A. flavus* that had been assigned to 8 vegetative compatibility groups. Protein extracts were electrophoresed on acrylamide gradient gels and isozymes were visualized by incubation with the appropriate reagents. Isozyme bands were numbered in the order of slowest to fastest migration and the presence or absence of each band was recorded for each isolate. Esterases ($\alpha$ and $\beta$) were found to be the most useful of 24 enzymes tested for rapid identification of specific members of *Aspergillus* section *flavi* as well as *Aspergillus* spp. Thirty-nine composite isozyme patterns were observed. While several isolates within the same VCG or species grouping had identical composite isozyme patterns, this did not occur with isolates in different species or VCGs. Thus, composite isozyme patterns could be used to identify species, the S and L strains of *A. flavus*, and specific VCGs.

The computer programs NTSYS-pc and PAUP were used to calculate pair-wise simple matching coefficients of similarity between all isolates for UPGMA cluster analysis, and for parsimony analysis. These data suggest the following:

There is esterase isozyme variation within species, however, the variability among species is much greater. *A. flavus* (S and L strains) and *A. oryzae* cluster together, as do *A. parasiticus* and *A. sojae*. The atypical S strain isolates of *A. flavus* are genetically distinct from the majority of *A. flavus* isolates. Esterase polymorphisms suggest *A. nomius*, *A. tamarii*, and *A. nidulans* are distinct from and ancestral to *A. flavus* and *A. parasiticus*.

Esterase isozyme pattern polymorphisms can be used to rapidly distinguish *Aspergillus* species, strains, and vegetative compatibility groups. The occurrence of consistent isozyme patterns within VCGs suggests VCGs are clonal lineages and that the sexual phase is either lacking or rarely occurs.

While UPGMA cluster analysis and parsimony analysis of esterase isozyme data generated dendrograms with differing key features, these data combined with molecular data (Amylase restriction site polymorphism data and genomic RAPD analysis) might be useful in addressing questions pertaining to the phylogeny and population biology of communities within *Aspergillus* section *flavi*.

POSTER

# Effect of Gossypol and Atoxigenic *Aspergillus flavus* Isolates on Sclerotial Production and Aflatoxin Biosynthesis by Toxigenic Isolates.

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Over the past five years we have observed sclerotia of the S strain of *A. flavus* on surfaces of tight locks in commercial cotton in western Arizona. Greenhouse experiments were conducted to assess variability among S strain isolates in ability to produce sclerotia in developing cottonseed and on surfaces of developing locules. All eight S strain isolates evaluated produced sclerotia on locule surfaces and 7 of 8 produced sclerotia within developing seeds. Apparently, the ability to form sclerotia on developing locules and within developing seeds is common among S strain isolates. Isolates varied, however, in magnitude and consistency of sclerotial formation. Sclerotial formation may play an important role in survival and dispersal of S strain isolates in cotton producing areas. Spindle picking may serve to scatter sclerotia on locule surfaces and seed containing sclerotia may provide reservoirs of nutrients for use after overwintering. High concentrations of aflatoxins, as well as several other potent toxins, occur in sclerotia. Previous researchers have reported that infected cottonseed contained higher aflatoxin levels when sclerotia were present than when seed contained no sclerotia. Thus, frequent formation of sclerotia within seeds and on lock surfaces may not only serve to facilitate strain dispersal and survival, but also to increase the toxicity and decrease potential uses of infected seed.

Gossypol, a toxic polyphenolic compound of plant origin, occurs in most cottonseed. During infection, *A. flavus* encounters quantities of gossypol often exceeding 1% dry kernel weight. Gossypol influences radial growth, sclerotial production and aflatoxin biosynthesis by *A. flavus.* Radial growth of both S and L strain isolates was inhibited by gossypol applied at a range of 20-2400 µg/ml in MES buffered Czapek's -Dox agar. Greatest inhibition occurred at pH 6.5 and 7.0. The presence of gossypol in the growth media (400 µg/ml, pH 7.0) stimulated production of sclerotia by L strain isolates. Conversely, production of sclerotia by S strain isolates was reduced in the presence of gossypol. Czapek's -Dox agar supplemented with 200 µg/ml of gossypol acetate enhanced the production of aflatoxin $B_1$ by both L and S strain isolates. The magnitude of the observed increase in aflatoxin production was substantially greater with the tested S strain isolate.

Certain atoxigenic isolates of *A. flavus* effectively reduce aflatoxin $B_1$ levels in cottonseed when coinoculated with aflatoxin producing isolates. These atoxigenic strains competitively exclude aflatoxin producing isolates during crop infection, and in so doing reduce aflatoxin contamination. Atoxigenic strains are being developed as biological control agents to prevent contamination. Coinoculation with atoxigenic L strain isolate AF36 resulted in both less aflatoxin in the mature seed and fewer sclerotia on locule surfaces and within seeds. The current study suggests isolates like AF36 may be particularly effective at excluding highly toxigenic S strain isolates. Ability to interfere with sclerotial production may further reduce the toxicity of infected seed and reduce S strain dispersal and overwintering.

## Management of aflatoxin contamination of cottonseed in Arizona

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The objective of our study is to develop optimal procedures to reduce aflatoxin contamination of cottonseed in Arizona. In 1994 we obtained additional evidence that D1, a bacterial isolate selected from among several hundred isolates, can both reduce Aspergillus flavus-infection of cottonseed and the level of A. flavus inoculum in the field and on cotton plant surfaces. Isolate D1 which is a strain of Pseudomonas cepacia, produces antibiotics effective against A. flavus, prevents sporulation of A. flavus in culture, on cottonseed, and on organic debris. The bacterium inhibits germination of sclerotia and reduces the level of boll rot both in the greenhouse and in the field.

The efficacy of D1 to reduce A. flavus infection of cottonseed was evaluated in field trials in Yuma, Arizona in 1993 and 1994. The level of A. flavus-infected cottonseed in plants spray-inoculated with a suspension of D1 was reduced by 90 and 81 percent, compared to that in untreated controls in 1993 and 1994, respectively. The differences between D1-treated and non-treated plants were statistically significant ($p < 0.05$) in both years. Spraying leaf, boll, and soil surfaces with D1 in the field resulted in reduction in the number of A. flavus propogules on these surfaces in both 1993 and 1994.

Results of 1994 ecological studies provided additional support for our earlier findings that isolate D1 can survive on leaf, boll, and soil surfaces in the field up to two weeks after its introduction. As in 1993, in 1994 populations of both D1 and A. flavus were significantly greater on boll surfaces than on leaf surfaces in the field.

Studies are under way to identify gene(s) mediating antagonistic activity of D1 against A. flavus and to increase antagonistic competence of D1 through genetic manipulations.

# Etiology of aflatoxin contamination in cottonseed from a subtropical environment

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Approximately 35% of the cottonseed arriving at the local cooperative oil mill exceeds a self-imposed action threshold of 15 ppb. This level of contamination results in thousands of dollars of lost revenue from sales of cottonseed meal for animal feed. The levels of aflatoxin contamination historically increase late in the harvest and in seed obtained from the upper Coastal Bend areas of Texas. In an attempt to identify primary sources of aflatoxin contamination, bolls were tagged at opening and harvested from several locations in the Lower Rio Grande Valley and upper Coastal Bend region during crop maturation. In addition to field plots which included reduced tillage and no-till treatments, cottonseed samples were taken from the Valley Oil Mill and modules from gins in both regions. Seed from various samples were screened for the presence of *Aspergillus flavus* and BGYF, subjected to radiography, and analyzed for aflatoxin contamination. Nearly 200 seed samples were tested for aflatoxin with only 10 showing any detectable level of the contaminant. Seven of the contaminated samples came from modules sampled within the same week from a lower valley gin. The levels of aflatoxin ranged from 30 to 4000 ppb and were limited to only two farms. The remaining three contaminated samples were replicates from a single harvest of lower bolls made at one of the Coastal Bend field plots. None of the samples from the Valley Oil Mill were found to be contaminated despite the detection of aflatoxin during routine testing of the incoming seed from a Coastal Bend area with a history of contamination. Radiographic analysis indicated that seeds from contaminated samples were incompletely developed and contained a larger number of empty seed. Poor seed development in other crops is typically associated with plant stress and may be associated with increased susceptibility in cottonseed. We were unable to confirm that lower (aging) bolls or seed from the Coastal Bend region are a primary source of aflatoxin contamination.

Over 2000 seed samples from single bolls to modules were screened for BGYF and the presence of *A. flavus*. These samples included tagged bolls harvested at different ages and positions from multiple field sites throughout the Lower Valley and Coastal Bend region near Corpus Christi, Texas. A. flavus was found in only 0.02% of the seed from older bolls in the lower positions on the plant. The fungus also was found in seed from newly-opened bolls but none of the seed from these samples exhibited any BGYF. BGYF seed were found in 0.02% of the samples from the modules. However, 75% of the BGYF seed were contaminated with *A. flavus*. *A. flavus* was found in only 1 out of the 7 module samples contaminated with aflatoxin. The incidence of *A. flavus*, BGYF, and aflatoxin was very low in the cottonseed samples tested. The lower or older bolls could not be identified as a major source of *A. flavus* or aflatoxin contaminated seed. There was no correlation of BGYF and A. flavus to aflatoxin contamination in the cottonseed samples. There did not appear to be a higher incidence of *A. flavus* and aflatoxin contamination associated with either the Lower Valley or Coastal Bend locations. The incidence of *A. flavus* and aflatoxin contamination was unrelated to the postharvest storage time in modules. The observed contamination in module samples was more likely a function of harvest date and producer location.

# SURVEY OF FACTORS INFLUENCING *Aspergillus flavus* INFECTION OF COTTONSEED IN SOUTH TEXAS

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The objectives of this study were to: (1) survey the incidence of *Aspergillus flavus* in cottonseed originating from two major cotton producing areas of south Texas and (2) identify factors that affect the severity of contamination. The survey consisted of: (1) individual bolls from field samples; (2) modules; and (3) ginned, aflatoxin-contaminated seed from Valley Coop Oil Mill. The fungus was assayed by plating surface-sterilized seed onto Czapek-Dox agar amended with 25 mg/L rose bengal, 50 mg/L streptomycin and 10 mg/L dicloran (AFIM). With boll samples collected from the field, infection of seed with *A. flavus* was low (0.1% incidence overall) and was not correlated with the presence of aflatoxin or BGYF. *A. flavus* was found in 9 out of the 12 fields surveyed, in both the Rio Grande Valley and in the Coastal Bend areas. The incidence of this fungus was 0.2% in bottom (older) bolls and 0.05% in upper bolls. There was no evidence that the incidence of *A. flavus* in bolls increased with the length of time that the bolls remained opened in the field. Indeed, relatively high incidences of infection were seen within a few days of boll opening as compared with several weeks of field exposure. For example, at the TAMU-Weslaco site, incidence of infection on newly-opened bolls was 0.3%, while that on bolls open for 48-54 days was 0.02%. There was no consistent relationship observed between type of tillage operation and the incidence of infection. There was no infection of insect-damaged bolls, but the sample size is likely too small to rule out insect damage as a contributing factor. With modules, *A. flavus* was found in 8/44 module samples. It was not found in any of 7 other samples which had > 10 ppb aflatoxin levels. In module samples, *A. flavus* was associated with BGYF seed. The frequency of BGYF seed in module samples was 0.02%. The frequency of *A. flavus* in BGYF seed was 75% (41/55 seed), while that in non-BGYF seed was 0.01% (1/8800 seed). The incidence of *A. flavus* and aflatoxin contamination was unrelated to the post-harvest storage time of the modules, which ranged from 1-18 days. There was no BGYF seed in any of the 10 samples of seed provided by Valley Coop Oil Mill. *A. flavus* was isolated from one seed from each of 4 samples.

# The Influence of crop rotations on soil surface populations of *Aspergillus flavus* in Arizona.

**Merritt R. Nelson[1], Donna M. Bigelow[1], Thomas V. Orum[1], Donald R. Howell[2], Stephen H. Husman[2], and Peter J. Cotty[3]**

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**Maricopa Agricultural Center Crop Rotation Experiment.**

The Maricopa Agricultural Center is a University of Arizona Agricultural Experiment Station facility located near the center of the cotton growing area of Arizona (right). The crop rotation experiment consists of sixteen 4 acre experimental plots with four treatments arranged in four blocks. Beginning in November 1992, a solum barley (winter)/ cotton (summer) rotation was established. The solum barley was harvested as seed followed by cotton with minimum tillage(treatment 1), plowed under as a green manure (treatment 3), or harvested as seed followed by late cotton using standard bed preparation techniques (treatment 4). Continuous cotton (treatment 2) is fallow during the winter. In 1994, sorghum replaced late cotton as the summer rotation in treatment 4. Soil samples (approximately 100 g from a 10 $m^2$ area) were collected from the soil surface (top 3-5 mm) at three sites approximately 40 m apart near the center of each experimental plot. The number of propagules of *A. flavus* per gram of soil was estimated from colony counts by dilution plating.

Variogram analysis (below) shows that there was spatial structure to the *A. flavus* soil populations on February 15 and June 6, because the sample variogram values (gamma) increase with lag distance over a range of 300 m. No spatial structure was detected in the October 14, 1993 sampling. Visual inspection of the field maps show that like-valued propagule counts were more clustered in February and June 1994 than in October 1993 (field maps below). The observed spatial patterns were not obviously related to the treatments. Analysis of variance of ranks indicated significant differences among the treatment means for both the October 14, 1993 and the June 6, 1994 sampling dates. It is too early in the experiment to tell whether consistent spatial patterns related to treatments will emerge.

**Commercial fields: Yuma County.**

In the August 1993, sixteen commercial cotton fields in Yuma County were selected for a study of the effect of crop rotation on soil populations of *Aspergillus flavus*. Fields were classified based on crops present in the field in October 1993 (Fall 1993 Crop), April 1994, and August 1994 (Summer 1994 Crop). Five soil samples were collected along a diagonal spanning each field. The number of propagules of *A. flavus* per gram of soil was estimated from colony counts by dilution plating. From the August 1994 samples, all colonies from selected dilution plates from each sample site in each field were subcultured for classification as L or S strains based on sclerotial formation. Between 40 and 80 colonies were classified from each field.

Analysis of variance showed significant differences in soil populations among fields in April and August 1994. In other words, the variance among the five sampling sites within fields was significantly less than the variance between fields. However, no significant differences among rotation treatments were detected by analysis of variance. Separate analyses were done using two crop classifications to define the rotations: the fall 1993 crop and the crop immediately prior to sampling. Results are displayed on the four maps below according to sampling date and crop classification (four maps below). Treatment differences shown are not significant statistically. Because the L strains generally produce less aflatoxin than S strains and because there is competition among strains for infection sites, a low percentage of L strains in a population may be a more important indicator of danger from aflatoxin contamination than high absolute soil populations. The percentage of L strain isolates differed significantly among fields, but those differences did not correspond to observed rotations (Map at right). Also, there was no correlation between percentage of L strain isolates and soil populations based on total counts of *A. flavus*.

POSTER

Aflatoxin Elimination Workshop
St. Louis, Missouri, October 24-25, 1994

# CHARACTERIZATION OF AN ASPERGILLUS FLAVUS POPULATION FROM A CORN FIELD IN CENTRAL ILLINOIS.

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The integrated pest management of A. flavus in corn cultivation requires that we identify sources of A. flavus inoculum within corn fields (i.e. soil, corn insects, air-spora, etc.) and determine which of these subpopulations are associated with aflatoxin contaminated grain at harvest. To accomplish this objective we used a molecular hybridization probe pAF28 containing a chromosomal DNA insert from A. flavus strain NRRL 6541 that is targeted to a repetitive DNA sequence in the A. flavus genome (McAlpin and Mannarelli, submitted). The probe is able to distinguish between strains of A. flavus belonging to different genotypes, including genetically isolated vegetative compatibility groups. An evaluation was made of the genetic diversity (DNA fingerprinting) of 211 A. flavus strains isolated from grain sampled at harvest (49 genotypes/ 70 strains), field soil (26 genotypes/ 31 strains), corn insects (49 genotypes/ 52 strains) and air-spora (56 genotypes/ 58 strains), from a corn field near Kilbourne, Illinois. Ninety four percent of the A. flavus genotypes produced sclerotia but only 50% of the genotypes produced aflatoxin. Contrasts of DNA fingerprints revealed two (2) matches involving subpopulations from grain and soil, one (1) match for grain and corn insects, and no matches for grain and air-spora. The high genotypic diversity recorded for each subpopulation, in addition to a a limited sample size, precluded any assessment of the relative importance of these subpopulations as sources of A. flavus infective inoculum. These results help to explain why we recorded only one A. flavus genotype infecting grain sampled in two or more years (1988-91) from the same 1.5 acre experimental planting. In 1991, DNA fingerprinting was performed on most of the A. flavus strains we isolated from grain sampled from each of 32 plots within this experimental planting. Aspergillus flavus genotypes, AF-IL#36 (aflatoxin positive) and AF-IL#47 (aflatoxin negative) were isolated repeatedly from two of the plots, evidence suggesting within-plot dispersal of an initial colonist by sap beetles, etc. Aspergillus parasiticus was recorded twice in kernel platings and was routinely isolated, along with A. flavus, from direct platings of field soil.

# Vegetative compatibility within populations of *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii* from a peanut field

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The diversity of vegetative compatibility groups (VCGs) in *Aspergillus flavus*, *A. parasiticus* and *A. tamarii*, mycotoxigenic fungi that produce aflatoxins and/or cyclopiazonic acid in peanut seeds, was examined. Soil samples were collected from a peanut field shortly after planting and peanut seeds were later harvested from the same soil sites. *A. tamarii* isolates were divided into morphologically distinct types A and B. A chlorate medium was used to select for nitrate-nonutilizing mutants (designated *niaD*, *nirA*$^-$, and *cnx*). VCGs were determined by pairing complementary mutants on a nitrate medium. VCG diversity, expressed as the number of groups divided by the total number of isolates, was in order of increasing diversity: *A. tamarii* type A (0.12); *A. parasiticus* (0.22); *A. tamarii* type B (0.31); and *A. flavus* (0.56). All isolates were incompatible in interspecific pairings; isolates of *A. tamarii* type A were also incompatible with type B isolates. Frequency distributions of VCGs from soil and infected peanut seeds were significantly different ($P < 0.0001$) for *A. flavus* and *A. parasiticus*. The high VCG diversity in *A. flavus* may be due to the influx of genetic variability through aerial spore dispersal from infected corn and cotton.

## Summary: Panel Discussion on Opportunities to Manipulate Microbial Ecology

The panel consisted of: J. Dorner, T. Isakeit, J. Dunlap, D. McGee, and I. Misaghi.
The discussion was chaired by P. Cotty who also prepared this summary.
Comments were also made by D. Wicklow, T. Michailides, R. Lynch, L. Jones, N. Keller, J. Radin, and M. Nelson.

Don Wicklow referred to the talk of Dennis McGee where McGee explained attempts to develop a model that would predict likely episodes of aflatoxin contamination. The value of such prediction may be to interfere with crop infection at or after bloom and Wicklow wondered how interdiction might be implemented. McGee responded that they first hoped to develop the model and determine what is actually going on and then work on practical interdiction methods. The two agreed that treatment of soils to affect *A. flavus* populations would be impractical. McGee suggested that some protection of silks may be possible.

McGee had also reported lower infection of silks in field plots in lines identified as resistant by Don White in Illinois. Peter Cotty questioned if silk resistance could be adapted to a laboratory screen to give breeders an additional trait to follow. McGee said they intend to pursue silk resistance further and determine whether true resistance was occurring or if they were observing an escape mechanism where silks of susceptible lines are merely susceptible for either longer or different periods. He referred to earlier work from Gary Payne's laboratory where green silks contained an inhibitor of infection as a potentially interesting lead.

Themis Michailides asked Cotty if the atoxigenic strains he applied had spread with predominant winds. Cotty indicated that the spread was not directional in the experiments he reported at the workshop. There were directional differences in the total amount of crop infection but, this may have been related to the amount of insect damage in different locations. Cotty indicated that dispersal may be more complex than just wind blowing conidia. Insects seem to be very real movers of *A. flavus*. Earlier reports by Tom Russell indicated a very high incidence of insects in cotton fields are associated with *A. flavus*. Michailides asked what insecticide applications were made to the plots. In the field plot experiments reported by Cotty, insecticides were applied to control white flies; these applications probably influenced other insects as well.

Michailides questioned Iraj Misaghi on his experiments utilizing bacteria to prevent contamination. He asked why Misaghi had sprayed the plants four times and if it related to Misaghi's bacterium not surviving well in the field. Misaghi indicated that the spray schedule was determined without knowledge of survival. The bacterium survived for the period between sprays, at least two weeks. Misaghi also indicated that the bacterium survived very well on boll surfaces.

Don Wicklow asked Joe Dorner how predation from mice or birds influenced atoxigenic strain rice inoculum in the field. Dorner indicated that he irrigates immediately after

application of the rice and as soon as the rice gets wet the fungus begins to sporulate readily. Thus, there is no time for predation or at least Dorner hasn't seen it. Dorner indicated that commercial situations may be very different and that he needs to develop application methods that ensure the applied material stays where it is needed. He also indicated an intention to empirically determine the amount of material he needs to apply. Cotty indicated that he has seen insect predation on the atoxigenic-strain-colonized wheat he applies.

Lynn Jones asked Joe Dorner if his atoxigenic strain applications influenced peanut quality or grade. Dorner indicated that peanuts from treated crops look just like peanuts from untreated crops. A very small percent is actually infected and/or contaminated. There are no differences in peanut grades caused by strain applications. Dorner indicated that there is still wild strain infection and some contamination (although greatly reduced) when applications are made but, the idea is to change the numbers game to allow acceptable contamination levels when atoxigenic strain applications are coupled with the other aflatoxin control strategies being developed.

John Radin asked Jim Dunlap why mills detected aflatoxins in cottonseed loads where Dunlap and Isakeit could not find *A. flavus* infection. A discussion followed, including Isakeit, Dunlap and many audience members, on problems in sampling caused by the low infection levels and highly variable aflatoxin content typical in aflatoxin contaminated commercial crops. There was disagreement on the relative reliability of commercial samples. Some felt the high level of variability meant a commercial sampling of 400 PPB did not necessarily indicate a high level of contamination in that load of cottonseed, others felt that a high level of contamination in a commercial sample indicated true existence of a contamination problem. Dunlap and Isakeit felt they were progressing towards a highly productive portion of the learning curve on the distribution of aflatoxin contamination in cottonseed in their area.

Cotty asked Dunlap what the incidence of unacceptable aflatoxin levels was in his area this year. Dunlap indicated that in a coastal area gin 25% of samples were unacceptable at the beginning of the season; this went up to 50% by the end of the season. In the Rio Grande Valley areas 5% unacceptable occurred at the beginning of the season and this increased to 20-25% by the end of the season. Dunlap felt he needed a very large sampling to determine the exact regional distribution of contamination; he indicated they may have a relatively small area with a high frequency of contamination. Isakeit indicated that there did not seem to be a relationship to storage in modules or rain on the mature crop and contamination.

Cotty mentioned that based on his experience in Arizona, the 0.5% and 0.05 % bright-green-yellow-fluorescent seed observed by Dunlap and Isakeit may be sufficient to cause unacceptable levels of contamination in the crop as a whole.

Merritt Nelson commented that pathogens with insect vectors have highly clustered patterns of distribution, assuming no secondary spread. Nelson asked if clustering had

been observed. Wicklow said his laboratory had certain field data that suggests clustering both of *A. flavus* infection and of specific genetic clones. McGee indicated that studies from his laboratory with waste corn showed such corn was heavily infested by nitidulid beetles. McGee said trapping experiments showed such beetles actually move inoculum out into and around the field.

Michailides asked if there are any real benefits to be gained from constructing genetically engineered fungal strains for competitive exclusion strategies. Cotty said he didn't know because he didn't have data on how such strains would perform in the field. There is considerable variability among atoxigenic strains in efficacy during competitive exclusion. Cotty indicated that the genetic technology is sound; as previously mentioned, once a strain is modified to lack a gene it has no potential to produce that gene product. He also commented that such strains may be useful for a variety of reasons, perhaps in reducing the allergenicity of strains.

Dunlap asked if *A. flavus* penetration into cottonseeds was necessary to get contamination or if contamination can come from bolls becoming infected without the seeds being infected. He also asked if the fungus needed cracking or some other avenue to get into the seed. Cotty mentioned that bolls inoculated after maturity can become infected readily upon exposure to adequate moisture and temperature; cottonseed at maturity is susceptible to contamination. McGee said that in general physical cracking is not required for pathogens to invade seeds; he suspects *A. flavus* can infect without cracks. However, cracking would increase seed invasion. Dunlap indicated that he was skeptical of infection of undamaged seed; he felt the literature implies some damage to kernels is required. Nancy Keller asked if cottonseed gets cracks like corn under stress. Radin indicated that cottonseeds have stomata and electron micrographs show these are generally open; stomata may be entry points for fungal hyphae.

Robert Lynch asked if the participants felt aflatoxin contamination could be controlled without controlling insects. He did not and Nelson agreed.

Dunlap mentioned that the portion of his area with the highest levels of aflatoxin contamination of cottonseed harvests cotton with strippers. This type of harvester collects a greater percent of insect damaged and partially opened bolls than spindle pickers. He wondered if this could cause part of the contamination problem. Cotty mentioned that tight locks and locules infected through insect wounds to developing bolls typically contain most of the aflatoxins. He further mentioned that the practice of collecting the cotton remaining after a spindle picker has harvested, with a rude, leads to increased contamination in Arizona. Rude harvested cotton typically contains high proportions of tight locules and insect damaged locules.

The final comment was made by Lynch who suggested that actual crop contamination might be increased with very minor insect damage. In peanuts, his laboratory had observed that only external scarification of the pod is required for insects to increase the incidence of contamination.

# CROP MANAGEMENT AND HANDLING

**Aflatoxin Elimination in Pistachio, Fig, and Walnut: Separation of Contaminated Nuts and Figs, Ecological Relationships, and Agronomic Practices.**

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Pistachios. Our objective was to determine if contaminated pistachio nuts could be separated from normal nuts in commercial processing plants using shell discoloration (staining). Nuts from two commercial processors were separated into various categories according to the external appearance of the shell. The kernels were examined for fungal infections and navel orangeworm (NOW) infestation (NOW infestation has been associated with high levels of aflatoxin contamination). No sign of fungal or NOW development were observed in nuts with no shell discoloration. Nuts with extensive (>one-eighth shell surface) dark brown shell discoloration had 29 and 9% fungal infection and 3 and 1% NOW infestation for the two processing plants, respectively. The nuts that had a limited dark brown (<one-eighth shell surface) could be separated into two distinct groups: those with discoloration along the suture (which had more than 18% of the kernels with mold and 1-6% infested with NOW) and those with discoloration on other parts of the shell (which had no kernel mold and no NOW). Over 35% of the nuts with an oily translucent appearing shell were infested with NOW. In addition, nuts that had yellow (but no dark brown) shell staining had 1% or less of the kernels contaminated with mold or NOW. Aflatoxin analysis of samples are in progress. Our preliminary results suggest that moldy pistachio nuts can be separated from normal nuts by differences in external shell characteristics.

Figs. Calimyrna figs were inoculated with *A. flavus* in a research orchard on four dates in August. Two weeks after inoculation figs were examined for infection by *A. flavus*. There was no clear trend for infections by *A. flavus* at the different inoculation dates. However, the figs became more susceptible as they matured through the four developmental stages (in parentheses are percentages of figs with external and internal *A. flavus* colonization, respectively): green with eye closed (0%, 0%), green with eye open (2%, 1%), yellow (15%, 11%), and brown (28%, 32%). In a laboratory experiment, green figs with closed eyes became susceptible to infection by *A. flavus* and *A. parasiticus* after wounding.

Although aflatoxin contamination does not seem to be a problem with some fig cultivars (such as Mission), aflatoxin contamination has occurred in fig cultivars besides Calimyrna, especially with the fig cultivar Conadria. These figs are distinct from Calimyrna figs in that they do not require pollination by wasps. Conadria figs were harvested from three commercial fig orchards on 19 August, 8 and 22 September. The percentage of figs infected with *A. flavus* group were 0.23, 0.20, and 0.20% for the three dates, respectively. Out of these figs infected with *A. flavus* group, only 11% had external lesions, while all of the rest had fungal growth restricted to the internal cavity. In addition, only 16% of these figs infected with *A. flavus* group were infested with the insect navel orangeworm. In one of these orchards, water from drip lines had rehydrated some figs so that 4% of them were infected with *A. flavus* group.

Walnuts. Preliminary studies were completed. Although the kernels from over 4,000 nuts were examined, no *A. flavus* or *A. parasiticus* were observed. However, the following types of nuts were associated with higher than normal levels of kernel infection by *Aspergillus niger* or other fungi: sunburned nuts, nuts infested with navel orangeworm, nuts on ground before harvest, nuts with earlier husk split, nuts with larger openings at the stem end, and larger sized nuts. Further studies are needed to determine if these observations are also true for *A. flavus* and *A. parasiticus*.

**The Relationship of the Date for Hull Split to Contamination of Pistachio Nuts by *Aspergillus* Species.**

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"Early split" pistachio fruits (ES), unlike normal fruits, split both hull and shell exposing the kernel to invasion by molds and insects. ES are undesirable because they are frequently contaminated with *Aspergillus* fungi, navel orangeworm, and aflatoxin. Although in early summer all pistachio fruits have the hull attached firmly to the shell, as the fruits develop, normally the hull detaches from the shell before the shell splits open. However, ES have the hull attached when the shell splits causing the hull to rupture. For each selected tree (in three commercial orchards), all pistachio fruits less than 2 m from the ground were carefully examined for hull splitting on 12 and 26 August and 7 September 1993. All ES were removed from the selected trees on 7 September, and the kernels were carefully examined using a dissecting microscope (10-60X) for sporulation by *Aspergillus* species and navel orangeworm infestation. Most ES (55%) had their hulls split between 12 and 26 August, although only 18 and 27% split their hulls before and after this period, respectively. ES that split before 26 August showed more than four times greater incidence of *Aspergillus* species than ES that split closer to harvest.

ES are probably the most important source of preharvest aflatoxin contamination for pistachio nuts in California. Although we did not measure aflatoxin, several factors that were observed have been associated with aflatoxin contamination. For example, navel orangeworm infestation of pistachio kernels has been associated with high levels of aflatoxin. The ES that split before 26 August had more than ten times greater infestation by navel orangeworm than the later-formed ES, probably because kernels were exposed to infestation for a longer period. Another factor associated with high levels of aflatoxin is shriveled hulls (in one study, ES with shriveled hulls had more than 99% of the aflatoxin detected). Most of the ES that split before 26 August had shriveled hulls at harvest time, while only 4% of the ES formed after 26 August did. Because the earliest-formed ES had the most decay by *Aspergillus* spp., the highest navel orangeworm infestation, and shriveled hulls more frequently, it would be expected that more aflatoxin would be found in these ES compared to ES formed close to harvest.

Many characteristics could be used to distinguish and remove the early-formed ES that are most likely to be contaminated with *Aspergillus*. The earlier-formed ES had lighter fruit weights and smaller shells compared to later-formed ES and normal nuts. In addition, the earliest-formed ES had hulls of less weight (fresh and dry) and less moisture content, shells with smaller widths, and kernels of less weight (fresh and dry) compared to normal nuts. In addition, the staining of the shell was greater the earlier the ES formed. Two types of external shell discoloration were distinguished. The first was the staining along the suture that is characteristic of ES, and the second was a general type of staining not localized in any one part of the shell. For both types of staining there were more staining with the earlier-formed ES compared to the later-formed ES and normal nuts. Shell discoloration would be the easiest character to implement by processors. By adjustments of electronic color sorters and instructions to hand sorters most of these early-formed ES can be removed.

***Aspergillus* Molds and Aflatoxins in Figs.**

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*Aspergillus* molds frequently infect and develop inside fruits of Calimyrna figs before harvest in commercial orchards in California. For each of eight orchards, 1000 and 2000 fruits were examined in 1992 and 1993, respectively. Twenty-two different *Aspergillus* species were found causing decay of figs. Although black-spored *Aspergillus* species (causal agents of the disease fig smut) were the most common species (occurring in 6.7 and 3.5% of the figs in 1992 and in 1993, respectively), there is a special concern for *A. flavus* and *A. parasiticus* because these molds produce aflatoxins. *A. flavus* was found in 0.05 and 0.04% and *A. parasiticus* in 0.01 and 0.02% of the figs in 1992 and 1993, respectively. Although some figs infected with *A. flavus* did not have any aflatoxin, other infected figs had high levels of aflatoxin (up to 24,000 and 9,600 ppb for *A. parasiticus* and *A. flavus*, respectively). Many of the *A. flavus* isolates from fig fruits did not produce aflatoxins in a glucose-yeast medium. It is difficult for processors to remove moldy and aflatoxin-contaminated figs because most of the infections are inside the fruit with no visible external symptoms.

Bright greenish-yellow (BGY) fluorescence under UV light (365 nm) was observed in 13 out of 16,000 Calimyrna figs examined. For these fluorescent figs, six figs had *A. flavus* sporulating in the internal cavity, while three figs had *A. parasiticus*. One fig with BGY fluorescence had *A. tamarii* (which is closely related to *A. flavus*). Only one of the BGY fluorescent figs showed the fluorescence externally, while for all of the others the fluorescence was observable only after cutting open the fig. Only one fig was found that had *A. flavus* growing in it but with no BGY fluorescence. Although there are problems with using BGY fluorescence (e.g., the need to examine the interior of the fig, not all contaminated figs fluoresce), BGY fluorescence might still be useful in some cases, such as fig paste processing.

Fungi in the *A. flavus* group were easily obtained from soil gathered in commercial fig orchards. A special medium was used that was selective for *A. flavus*: 10 g sucrose, 1 g yeast extract, 60 g NaCl, 0.1 g chloramphenicol, 10 ml dichloran solution (0.2% in ethanol), 15 g agar, 1 L distilled water. After 0.5 g soil was sprinkled on a petri plate with this medium, the plate was incubated at 37C for 4-5 days. *A. flavus* strain L was found more frequently than any other aflatoxin-producing species or strain. Fortunately, many isolates of *A. flavus* strain L do not produce aflatoxin. *A. flavus* strain S (produces abundant small sclerotia in culture) was also found in several orchards. In 1993, five out of the nine orchards had more *A. flavus* in their soil than *A. parasiticus*, whereas the rest had more *A. parasiticus* than *A. flavus*. There were large differences in the levels of these fungi for different orchards. For example, in 1993 the number of colony forming units per gram soil detected for *A. flavus* ranged from 0.5 to 30.8 for different orchards with a mean of 5.8 and for *A. parasiticus* ranged from 0.0 to 9.7 with a mean of 3.2. *A. flavus* was found in every orchard, while *A. parasiticus* in all but one of the orchards. This result suggests that the potential for aflatoxin contamination exists in every Calimyrna fig orchard.

Insect IPM for Mycotoxin Control In Midwest Corn: 1994 Studies

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Different malathion formulations were tested for effectiveness in controlling corn insects (and associated ear molds) in small plot trials. Conventional application techniques and technical grade material were used. Formulations consisted of single and multiple conventional sprays (low rate of 0.1 lb/A a.i. and normal rate of 1 lb/A a.i), and single treatments of adherent granules (ultralow, low and normal rates of 0.01, 0.1 and 1 lb/A a.i., respectively) and starch sprays (low rate of 0.1 lb/A a.i. and normal rate of 1 lb/A a.i.). Based on numbers of dead and live sap beetles seen in leaf axil samples, control was best with normal and low adherent granules rates and 5 normal rate sprays. Milk stage ear samples indicated corn rootworm adults were only significantly controlled (by 43%) with the 5 normal rate sprays. Compensating for first application time coverage (57% silk), caterpillar control with ultra low and low granule rates would be comparable to the 5 normal rate sprays (34, 40 and 71%, respectively). Predator abundance was reduced by the 5 normal rate sprays and the normal rate granules. At harvest, *Fusarium* spp. mold was reduced by 34% with the 5 normal rate sprays and by 40.5% with the normal rate granules. European corn borer stalk tunnel lengths were not affected by the 5 normal rate sprays, but were reduced by 30-35% with the granular treatments.

Additional small plot trials involved treating a commercial corn line moderately resistant (R) and susceptible (S) to *Fusarium* spp. ear molds (both Pioneer) with a single treatment of 0.1 lb/A a.i. adherent granules. Sap beetles occurred at much higher levels (ca. 10 X) in axels of the R vs. S lines, but were controlled by 54% with the granules. Predator levels were unaffected by the granules. Based on sizes of larvae found, milk stage samples indicated ears were protected by 2.5 fold for 10-14 days against European corn borers by the granules. At harvest the number of kernels with *Fusarium* was reduced by 50% in the R line, while 40% fewer ears of the S line had *Fusarium* when treated with granules. Obvious sap beetle damage of ears at harvest closely corresponded to distribution seen with axil samples.

With representative lines R and S to aflatoxin and *F. graminearum*, (provided by CIBA seeds), incidence of caterpillar survival was about the same (75-95%), but caterpillars were ca. 20% smaller on the R lines after 14 days. Natural populations of sap beetles infested the R caterpillar damaged ears by 4-10 X less compared to the S ears, and when added to exposed kernels, damaged 1.5 to 2 X less kernels in the R vs. S ears. The R kernels were ca. 1.6 X more resistant to penetration by abrasion.

Similar to results reported for oxidative enzymes of corn involved in resistance (J. Chem. Ecol. 20:2497), kojic acid inhibited oxidative enzymes of fall armyworms involved in pathogen resistance (ca. 80% at $10^{-3}$ M and 40% at $10^{-4}$ M), indicating a dual function. At 1000 ppm, 4-ABOA was less active than MBOA to corn earworms, European corn borers, and adult sap beetles; but more active than MBOA to fall armyworms. Cyanidin had no significant effects on corn earworms at up to 2% (20,000 ppm). Although alone they had little effect, linolenic, chlorogenic, ferulic, sinipic and caffeic acids combined with horseradish peroxidase inhibited hyphal elongation of *Fusarium graminearum*, but not *Aspergillus flavus* in *in vitro* assays. The degree of effect was dependent on the concentration of the substrates and enzyme.

Additional tests with autoinoculators indicated sap beetles would carry commercial *B. subtilis* (Kodiak) to corn, as it was reisolated from corn visited by sap beetles in field cage and field trials. Using only 1 autoinoculator in a 2 acre area, and considering unmolded kernels as having < 200 ppb aflatoxin, 75% of ears denied access by sap beetles contained damaged kernels with > 200 ppb of aflatoxin, while only 7% of ears did where beetles were allowed access. From 90 to 100% of perimeter traps around 40 acres with the single autoinoculator captured sap beetles carrying *B. subtilis* after the completion of the experiment. Large vials (ca. 90 ml) holding sap beetle attractants lasted for at least 3 months without changing, vs. 3 weeks for previously used small vials (5 ml); attractancy was comparable to frequently changed attractants.

# Aflatoxin in 1994 South Georgia Corn: An Expert System to Aid in its Control

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The 1994 South Georgia corn crop realized the least severe contamination by aflatoxin of any year since records were begun in 1977. In the 1994 survey of 36 counties, the average level of contamination was 6 ng $g^{-1}$ with the most contaminated sample having 50 ng $g^{-1}$ aflatoxins. The low contamination can be attributed to environmental conditions that were excellent for growing corn throughout the growing season. Stress due to shortage of moisture was minimal.

The fields of six farmer cooperators within a 75-mile radius of Tifton were monitored for as many as 33 environmental and sample variables to establish relationships with aflatoxin contamination and Aspergillus infection percentages. Two locations were monitored as dryland corn, one as irrigated corn, and three under both dryland and irrigated conditions. In spite of very low levels of aflatoxin contamination in samples at all locations, the trends found for relationship among variables were similar to those found in 1993; that is, canopy relative humidity, ear temperature, and ambient temperature were variables most closely related to aflatoxin contamination, while canopy temperature, ambient temperature, solar radiation, and ear temperatures were most closely associated with A. flavus ear infections.

The expert system being developed for minimizing aflatoxin contamination of corn grown in the South will consist of the integration of three principal components: 1) the body of knowledge for growing corn in the southern states to maximize profit through optimum yields; 2) the body of knowledge for management practices that minimize aflatoxin contamination of corn grown in the southern states; and 3) the information accumulated from collection of environmental data in this study to be used in the modification of principles deduced from the bodies of knowledge listed above. We are developing a system with several modules to avoid requiring the grower to follow flow charts through the whole system each time he wants information. For example, he would have no interest in the planning, harvesting or storage modules when he was only trying to determine if irrigation were needed. Flow charts have been completed for five of the seven or eight needed to complete the system.

# Investigations of the Influence of Modular Storage on *Aspergillus flavus* Infection of Cotton seed and Aflatoxin Contamination

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Samples of seed cotton from thirty fields in the Mississippi Delta were assayed for superficial populations of *Aspergillus flavus* in 1993 to ascertain the extent of seed cotton contamination and to identify appropriate sites for further study. *A. flavus* group fungi were recovered from seed cotton rinsate from 14 (47%) of the 30 sites sampled. Only two of these sites were among the seven (23%) of 31 sites where *A. flavus* was found in 1992. Seven other 1992 sites were positive for *A. flavus* group fungi when sampled again in 1993. Five additional sites of *A. flavus* contamination were identified in 1993. Populations in 1993 were however extremely low, generally below 225 propagules/gram of seed cotton, except for one site where populations exceeded 1700 propagules/gram of seed cotton. Additionally, fuzzy seed samples were obtained from 106 modules during ginning at the Hope Gin at Thornton, MS. Of these, *A. flavus* infected seed were found from 16 (15%) of the modules. Based on the 1993 field survey, modules were constructed near Shelby, Gunnison, Panther Burn, and Sidon, MS and monitored weekly for moisture, temperature, and aflatoxin. Moisture and temperature of seed cotton were within acceptable ranges and were indicative of a lack of microbial activity. Not unexpectedly, since superficial populations of *A. flavus* had been low on seed cotton, aflatoxin contamination of seed was rare and when present below 2 ppb. However. aflatoxin was identified from each of the four modules studied. Sampling ports proved an appropriate avenue for repeated sampling.

Low populations of *A. flavus* on seed cotton and low levels of seed infection prior to moduling have not provided a suitable module for the study of the influence of modular storage of seed cotton on aflatoxin contamination. Consequently, in an effort to obtain a more highly contaminated crop, bolls in a field of cotton were artificially inoculated with a spore suspension of *A. flavus*. Two modules have been constructed from this crop and are currently being sampled. Samples from the module surface are to be processed as well as those from the module sampling ports.

In summary, *A. flavus* contamination of Delta-grown cotton does not appear to be uncommon. However, populations of the fungus have been relatively low. Aflatoxin contamination of seed in modules built from seed cotton having these low populations of *A. flavus* has been rare and when present has been of low concentration. However, all modules sampled have been positive for aflatoxin.

Batson, W.E., Jr. and D.H. Newman. 1993. Effect of modular storage on the incidence of *Aspergillus flavus* contamination of seed cotton in Mississippi in 1992: A preliminary report. Proc. Beltwide Cotton Prod. Res. Conf., Cotton Disease Council 53:209-210.

Batson, W.E., Jr., J. Caceres and Debra Newman. 1993. Influence of modular storage on *Aspergillus flavus* contamination of seed cotton in Mississippi. Phytopathology 83:1418.

Batson, W. E., Jr. and J. Caceres. 1994. Aflatoxin development in modules during field storage in the mid-south. Proc.Beltwide Cotton Prod. Res. Conf., Cotton Disease Council 54:279.

# EVALUATION OF THE EFFECT OF LIGHT, STERYL-FERULATES AND OTHER FACTORS ON *A. FLAVUS* USING A SUSPENDED DISC CULTURE SYSTEM

Aflatoxin Elimination Workshop--St. Louis, MO, October 23-25, 1994

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ABSTRACT

A new method of growing *Aspergillus flavus* for experimental studies was used to evaluate the effect of representatives of three different groups of compounds which are found in the corn pericarp/aleurone layers. The culture system consists of a humidified vial with a thick septum pierced by a pin on which a glass fiber disc is affixed. The disc contains the test solution, inoculum and medium. Before using the method for testing compounds a number of parameters which could affect aflatoxin (AFT) $B_1$ levels were investigated. Using *A. flavus* NRRL 6536, it was found that AFT $B_1$ level was affected by method of inoculating the disc, the amount of medium placed on the disc and type of disc material. Gelman Sciences Extra Thick Glass Fiber (ET) disc gave the best performance for toxin yield and variability if the disc material was pretreated by washing in a series of organic solvents (acetone, benzene, chloroform and methanol). There was a 14-fold increase in AFT $B_1$ for ET discs compared to 0.5 ml vial culture. Discs with medium at levels from 139% to 56% of disc saturation gave AFT $B_1$ levels from 4.94 ug/ml of medium (139%) to 10.6 ug/ml (65%). Medium at 86% saturation was used for most subsequent studies. Discs as small as 6mm, using 29ul of medium, gave acceptable statistics (15% CV) when used with *A. flavus* NRRL 3357.

In examining factors which might cause high variance in results with #6536, the effect of cool white fluorescent light was investigated. Cultures of #6536 given 15 min/day laboratory light averaged 8 times more AFT $B_1$ than dark controls. Cultures given 3.75 min/day light had ca. 4 times more AFT $B_1$ than dark controls--maximum AFT $B_1$ was produced at 7.5-15 min/day for #6536. NRRL 3357 showed a lower sensitivity to light; maximum effect was at 1 hr/day which produced 2.1 times more AFT $B_1$ than dark controls. The problem with variability led to examining other lines for use with the assay. Use of *A. flavus* NRRL 3357 was found to give much lower variation (typical CV 5-10% compared to 30-50% for #6536); it also produced ca. 8 times more AFT $B_1$ than #6536.

Fractions or compounds of three groups of secondary compounds occurring in corn pericarp were tested for effect on growth and AFT $B_1$ production using either line #6536 or #3357. The total steryl ferulate/*p*-coumarate fraction of corn bran was tested, using 1% PC as a solubilizer, at levels from 0.1 to 3.33 mg/ml of medium. At 1.0 mg/ml the ester fraction stimulated AFT $B_1$ production to 1.9 times the level of the control; at 0.33 mg/ml, the estimated level in the pericarp, AFT $B_1$ was 1.6 times the control. When tested against #3357 the esters had no appreciable effect. PC solutions over the range 0.1-5% were tested for effect on toxin production; a 0.7% solution of PC with medium gave a 4-fold increase in AFT $B_1$ with #6536. A 0.1% level was 187% of the control and a 5% solution was only 21%. Three members of the benzoxazolinone group, which occur in corn, were tested. These compounds are soluble in acetone but not water. All were tested at levels of 0.016, 0.08, 0.4, and 2.0 mg/ml. 4-Acetybenzoxazolinone (4-ABOA, provided by courtesy of David Miller of Agriculture Canada), which occurs in the pericarp , strongly depressed AFT $B_1$ production with little effect on growth except at very high concentrations of 4.5 and 9.0 mg/ml. At 0.4 mg/ml toxin production was reduced to 23.3% of the control. 6-Methoxybenzoxazolinone (MBOA), which occurs in vegetative parts of corn, showed a complete inhibition of growth and toxin production at 0.68 mg/ml, a reduction of 45% at 0.4 mg/ml but only 8.5% in growth. Benzoxazolinone (BOA), which also occurs in the vegetative portions of the plant, showed sharp, parallel declines in both AFT $B_1$ and growth at levels of 0.016 mg/ml and 0.08 mg/ml with no growth or toxin at 0.4 mg/ml. Cyanidin, the aglycone of several anthocyanin pigments which occur in the pericarp/aleurone, was tested at levels of 1.25, 5.0, and 20mg/ml. The pigment is water soluble. No effect on AFT $B_1$ was seen at 1.25 mg/ml but 5.0 and 20 mg/ml inhibited toxin by 37% and 87%. Growth was increased by 12% at 20mg/ml and decreased by 18% and 9% at 1.25 and 5 mg/ml.

When used with a suitable *A. flavus* line such as NRRL 3357, the suspended disc culture method yields results with variability equal to or less than liquid vial cultures and allows highly hydrophobic compounds, such as steryl ferulates, to be tested with solubilizers which do not depress aflatoxin production and are normal components of corn kernels. Compounds with intermediate solubility, such as benzoxazolinones, can be incorporated into the discs in an organic solvent which is then evaporated and the disc inoculated with a medium/spore mixture. For aflatoxin control, 4-acetylbenzoxazolinone is at least one promising kernel-associated compound. This compound was able to significantly depress AFT $B_1$ synthesis by *A. flavus* at physiological levels.

POSTER

## Machine vision system for automated detection of stained pistachio nuts

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It is desirable to remove stained pistachio nuts because they have lower consumer acceptance and higher incidences of aflatoxin contamination. The pistachio industry currently utilizes a variety of methods and equipment to remove stained nuts. Automatic color sorters are used to remove badly stained nuts. However, many unstained nuts have wide shell openings, exposing more of the dark kernel. This causes the average nut color to have a more reddish hue. As a result, the nut might be rejected by the automatic color sorter. Conversely, some nuts accepted by the automatic color sorter have small stains with insufficient area to affect the average nut color.

HPLC aflatoxin analysis was performed on a set of small shelling stock nuts collected from a California pistachio producer. The nuts were split into four classes: long and stained, short and stained, long and unstained, and short and unstained. Each class contained ten samples of 100 nuts. A nut was considered stained if more than 10% of its shell area was discolored and long if its overall length from stem to calyx end was greater than 15.5 mm. These tests determined that nearly all of the aflatoxin is concentrated in the stained nuts. Only one sample from the twenty pooled unstained samples tested positive; and at a very low level, 0.09 ppb. Seven of the twenty pooled stained nut samples tested positive. Three of the positive samples were at low levels (less than 0.18 ppb); but, four samples had concentrations of 1.45 ppb, 3.61 ppb, 10.87 ppb, and 421.17 ppb. The average aflatoxin concentration in the twenty nut samples was 21.9 ppb in the stained nuts and 0.005 ppb in the unstained samples.

The machine vision system was built by modifying a monochrome sorter (ESM, Microscan II, Houston, TX). This device uses three line scan cameras (256 pixels/line, 200 KHZ pixel rate) to scan the entire perimeter of the nut. The ESM sorter was modified by diverting the output of each camera is to a digital signal processing (DSP) board hosted in a 486-33 personal computer (PC). In the video signal, unstained portions of the nuts have low intensity and low gradient (change in intensity from pixel to pixel) values. Pixels representing stained portions of the nut have higher intensity and gradient values. The DSP counts the number of pixels with both a high intensity and gradient, number of pixels with both a low intensity and gradient, and also computes the number of regions in the video signal with high intensities and gradient values. This third parameter indicates the number of stained and unstained regions on the nut. These three parameters are combined on the PC after the nut passes out of view from the camera. Discriminate analysis was used to develop discriminate functions to determine if a nut is stained or unstained. Separate discriminate functions were developed, for both the automatic color sorter reject and small shelling stock process streams, using data from 300 stained and 300 unstained nuts from each process stream. The PC uses the discriminate functions to compute the probability of the nut being stained. Depending on this probability, the PC can activate an air nozzle to reject the nut.

This machine vision system has an overall minimum error rate of 14% for automatic color sorter reject nuts, and 15% for small shelling stock nuts. The maximum throughput rate is 40 nuts/s. The cost of this system is estimated to be about $10,000 and all of the hardware is available as off the shelf items. Other pistachio process streams and possibly other commodities might be inspected by this system with modifications to the software.

Distribution of Aflatoxin in Processed Pistachios. Thomas F. Schatzki and James Pan.
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The distribution of aflatoxin in 17 process streams was measured. These process streams resulted from commercial sorting of bin-stored pistachios by a California processor of the 1992 crop. Measurements were made on 20 samples each of 10, 100, and in most cases 1000 nuts. It was found that aflatoxin was largely concentrated in low quality product, consisting of small nuts [26 or more nuts/ounce] and in discolored floater nuts. A removal of 4% of the product can achieve an elimination of 88% of the current aflatoxin level. Similar results were subsequently found by another processor, using a substantially similar process for the 1993 crop. However, in the latter case the use of the color sorters to select a much smaller reject cut [1.2% versus 13%] resulted in a smaller reduction of aflatoxin content. It is thus essential to remove any questionable nuts from the process stream. Work is in progress to develop additional sorting methods which may allow return to commerce of part of the reject streams. If successful, this will reduce the 4% product loss even further without significantly increasing overall aflatoxin content.

Measurement of aflatoxin distribution over a range of sample sizes allows one to express the frequency of contamination as a function of contamination level over an 8-decade range [from 0.01 ppb to 1,000,000 ppb]. One finds that this frequency can be expressed as 0.03/sqrt(c), where c is the concentration of aflatoxin in a single nut in ppb [roughly ng/nut] and the frequency applies to reject process streams. It has been established that nuts containing aflatoxin in excess of 10,000 ppb are the main cause of samples failing U.S. standards. Such nuts are now seen to occur less frequently than 1 nut per 30 lbs. in low quality product and less than 1 nut per 300 lbs. in high quality product. As a result, product testing for aflatoxin is notoriously difficult in pistachios and other tree nuts. The results obtained here suggest that the reduction of aflatoxin in U.S. product can be controlled more easily by process control than by product testing. This concept is being incorporated into a proposed marketing agreements for pistachios. The reduction of overall level will also open a wider foreign market for U. S. produced pistachios.

POSTER

# MACHINE RECOGNITION OF NAVEL ORANGE WORM DAMAGE IN X-RAY IMAGES OF PISTACHIO NUTS

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Insect infestation increases the probability of aflatoxin contamination of pistachio nuts. Manual inspection is used to remove nuts with external evidence of insect infestation just prior to roasting. An automated inspection technique is needed to improve completeness and consistency of insect removal, remove infested nuts which do not show external evidence of infestation, and reduce costs of manual inspection. X-ray film images reveal evidence of insect infestation which can be readily recognized by a trained observer. The objective of this work was to develop an X-ray based automated inspection method for insect damage in pistachio nuts. Images of 189 infested and 270 insect-free pistachios were obtained from a linescan X-ray machine with 0.5 mm sensors, and from x-ray film scanned at 0.125 mm/pixel. Film images were further reduced to 0.25 and 0.5 mm/pixel by pixel averaging. All images were convolved with a difference of gaussians (DOG) filter. Image features were calculated from the intensity and DOG images. For each input image (2 per nut), histograms of derived intensity, curvature and contrast images were used to compute statistical moments (area, mean, variance, skewness, and kurtosis), 30 moments or features per nut. These were then used as the input patterns for development of discriminant functions and neural networks. Good recognition was achieved with high resolution images but deteriorated with decreasing resolution and increasing noise. Recognition results (% recognition of infested nuts, % misclassification of clean nuts) were: 81%, 5%; 78%, 7.4%; 79%, 14%; 67%, 18% using 0.125 mm, 0.25 mm and 0.5 mm/pixel film images and 0.5 mm linescan images respectively.

POSTER

## Effect of Strains of *Aspergillus flavus* on Selection of Corn Genotypes for Resistance to Kernel Infection and Aflatoxin Contamination

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*Aspergillus flavus/parasiticus* kernel infection and subsequent aflatoxin contamination continues to be an important economic problem in some corn growing areas in the United States. Corn grain containing in excess of twenty parts per billion of aflatoxin can not be sold in Interstate Commerce. Current research demonstrates that *A. flavus* can infect intact kernels directly through the pericarp in the field before harvest. Injury to kernels in the field by several species of corn insects can increase kernel infection and aflatoxin accumulation. However, kernel infection by *A. flavus* and subsequent aflatoxin accumulation can occur in the absence of insect damaged kernels.

Part of the problem with selecting corn genotypes with resistance to *A. flavus* infection and aflatoxin accumulation is that *A. flavus* is a weak parasite that causes little, if any, visual damage to kernels or reduction in yield. Assaying corn grain from inoculated ears for kernel infection or aflatoxin contamination is difficult and expensive. Often, indirect methods must be used. There is also the question of whether to assay for percentage kernel infection or for aflatoxin content using immature or mature kernels, or to test damaged or undamaged kernels. Because of variability in kernel infection on individual inoculated ears, obtaining a truly representative grain sample for assay is sometimes difficult.

Assaying a kernel sample from a test plot for aflatoxin using the fluorometric technique takes about 15 minutes. Assaying the same sample for percentage of kernel infection takes longer and requires one week of incubation. In our laboratory, the cost of assaying for both methods using part-time student labor is similar.

The variability among isolates of *A. flavus* on corn is well demonstrated. Individual isolates of the fungus have been identified with relatively high or low aggressiveness in relation to kernel infection. Other isolates are known that are high, low, or non-aflatoxin producers. Some isolates, such as NRRL 3251, are not very aggressive but produce high levels of aflatoxin in infected kernels. On the other hand, NRRL 3357 produces moderate levels of aflatoxin in infected kernels, but is more aggressive than NRRL 3251. The identification of these isolates raises the question of which assay method (percentage kernel infection or aflatoxin accumulation) should be used for selecting corn lines for resistance to *A. flavus*.

There is good evidence that resistance to kernel infection by the fungus and subsequent aflatoxin accumulation in corn, although affected by individual fungal

isolates, is genetically controlled. Three corn genotypes, Mp313E and Mp420 from Mississippi and GT-MAS:gk from Georgia, have been identified as sources of resistance to kernel infection and aflatoxin accumulation.

We feel that initially assaying kernels from field inoculated ears for resistance to aflatoxin accumulation, followed by assaying promising genotypes for resistance to kernel infection, has promise for developing corn genotypes resistant to kernel infection and aflatoxin contamination by *A. flavus*.

**Summary: Panel Discussion on "To What Degree Can Optimum Crop Production and Handling Practices Eliminate Aflatoxin?"**

The panel consisted of P. Dowd, N. Widstrom, W. Batson, T. Schatzki, R. Norton, and N. Zummo. Michailides chaired the discussion and wrote the summary. Comments were also made by P. Cotty, M. Doster, T. Isakeit, C. Martinson, and D. Wicklow. Although their names were included in the initial schedule, J. Dunlap and N. Goodman did not participate in the panel discussion.

Because there was no time for specific questions after each presentation, the chair asked that specific questions related to the material presented be directed to the presenters first, after which the discussion would be open to more general matters relating to the topic of the panel.

Cotty asked Michailides if he had analyzed any figs for aflatoxin which did not show growth of *A. flavus* or *A. parasiticus*. Michailides stated that he had not. Cotty suggested that aflatoxin can develop even without obvious growth of *A. flavus* and Michailides agreed. Michailides also mentioned that it was unlikely that even a small growth of *A. flavus/parasiticus* would not been detected since all figs were split in half and examined with a dissecting microscope. Cotty also asked Michailides if there was a possibility of *A. flavus/parasiticus* growing in the flesh tissues of figs without invading the cavity. Doster reported that the high relative humidity in the cavity favors the growth of *Aspergillus* spp. (including *A. flavus/parasiticus*) in the cavity rather than in the spongy tissues of the syconium walls. Michailides added that the presence of the oily seeds in the cavity in some varieties will also help in the growth of *Aspergillus* spp. in the fruit cavity.

Wicklow asked Michailides if he had isolated *A. flavus* or other microorganisms from the inside surface of the ostiole of figs. Michailides stated that he did not do this, although he believed he would find almost any microorganism (including *Aspergillus* spp.) that is present in fig orchard soil to be present in the ostiole, too. He made this comment because he indicated that when figs are split in half he could find significant amounts of soil in the area around the ostiole. However, because this area (around the ostiole) dries quickly, the microorganisms which are present do not grow. In addition, a dense layer of waxy scales present in the inner surface of ostioles may prevent the growth of these microorganisms on the ostiole.

Michailides asked Widstrom whether the "expert system" he is developing will take into consideration the various parameters as 'yes' or 'no' decision-making statements or as quantitative parameters with 'low,' 'medium,' or 'high' levels. Widstrom explained that since there is voluminous information on aflatoxin research of corn his expert system will be similar in a way with that developed and used in peanuts. Most of the decision-making parameters will be entered in the flow charts of the computer program as 'yes' or 'no' answers.

Martinson questioned Widstrom further on the type of information that would be given in their "expert system." Widstrom summarized his answers as follows: The expert system being developed for minimizing aflatoxin contamination of corn grown in the South will consist of the integration of three principal components: 1) the body of knowledge for growing corn in the

Southern states designed to maximize profit through optimum yields; 2) the body of knowledge for management practices that minimize aflatoxin contamination of corn grown in the South; and 3) the information accumulated from collection of environmental data in this study to be used in the fine tuning and modification of principles deduced from the bodies of knowledge listed above. He said that they are developing a system with several modules to avoid requiring the grower to follow flow charts through the whole system each time he wants information. Widstrom added that assessing economic losses or gains resulting from management practices generated by the system was turning out to be the most difficult aspect of system development.

Michailides asked Schatzki if he could define better (or give more details) on the '4% other damage' in pistachios he had sampled from processing plants as he had presented in his talk. He stated that pistachio early splits represent about 2% of the crop. Michailides mentioned that his laboratory had orchard data where counts of thousand of nuts in the field showed that sometimes early splits can be 5% or higher. He also mentioned that in last year's research they (Doster and Michailides) showed that 99.9% of the aflatoxin that was measured in their samples was found in shriveled early splits and nuts infested by the navel orangeworm. The components that constituted the '4% other damage' as it was defined by Schatzki remained unclear.

Schatzki extended his remarks by saying that Sommer selected 2% of nuts (identified by him as early splits) and his distributions are based on that selection. Additional nuts of poor appearance were left on the trees because it was felt that such nuts would not enter the process stream (Sommer, *personal comment* 1992 or 1993). In fact, when shaken down nuts were examined by Schatzki and coworkers, 2% showed the classical early split hull while another 4% were crinkled, black, etc. (These might well have had early split hulls and/or insect/bird damage). The point at issue is what fraction does Sommer's distribution represent of the total product stream entering the processing plant. Put another way, by what factor does Sommer's distribution need to be multiplied to get a distribution representing the entire shake down crop? If the remaining 94% are clean (as they largely are from our data) and the 4% have a distribution similar to that in Sommer's data (as they did in Schatzki's data) then the answer is 0.06. This is the factor we used, he said, and these results `are` an excellent fit between Sommer's data x 0.06 and the DFA data for 1983-1986 and for 1990-1991.

Wicklow asked Dowd what progress has been made in licensing the adherent granular formulation of malathion. Dowd indicated that there has been back and forth discussions between ARS and member companies of the Biotechnology Research and Development Corporation (who have provided additional funding to M. R. McGuire) as to who should be responsible for dealing with the E.P.A. Dowd added that since additional funding has been received (by M.R. McGuire and ERDC) from the Agricultural Research Consortium to develop this technology, the increased visibility would hopefully stimulate the process. (Since the workshop, a tentative decision has been made to hire an independent consultant to do the paperwork on this project.)

Wicklow asked Batson what he really meant by saying that few samples of cotton seed sampled from modules in the Mississippi Delta had small amounts of aflatoxin. What were these small amounts? Batson answered they had rarely detected aflatoxin and, when present, it was below 2 ppb. Wicklow then added that it seemed that there was indeed no aflatoxin problem in the

Mississippi Delta. Cotty pointed out that although the industry did not have an aflatoxin problem now they wanted to determine whether aflatoxin would ever be a problem in Mississippi.

Cotty asked Dowd if host relationships for sap beetles were well known. Dowd responded that in many cases they were. Much of this work (host relationships) had been done with corn in the midwest and northeast. The most common sap beetle in corn overall is the dusky sap beetle. Its range is from the northern United States south through Brazil. It moves from decaying materials available in the spring and early summer, such as corn residue, to corn, feeding on pollen in axils, and then moves into the ears as kernels develop. Many other beetle species also feed on corn at one time or another. Dowd then referred to Michailides for comments on those invading fruit.

Michailides added that nitidulid beetles (such as *Carpophilus hemipterus* or *C. freemanii*, etc.) are a major problem in California stone fruits and figs and become very abundant particularly when fruit mature. They can find them very often on decaying fruit both on trees and on the ground. The beetles can vector brown rot (*Monilinia fructicola*) spores from fruit to fruit. These beetles can be found easily during the winter in remnants from fruit on the orchard floor. It seems that they survive as adults on remains of decayed fruit and probably are hiding in the soil. If figs are planted close to stone fruit orchards, nitidulid beetles will be a major problem since they also prefer figs. Michailides stated that his station has an experimental fig orchard in the field of the Kearney Agricultural Center next to a nectarine/peach/plum/apricot orchard; in this orchard, they have always had problems from nitidulid beetles, mainly after figs became yellow and the ostiole opened. Although the fig industry is located now in Madera and Merced counties far away from stone fruit orchards, nitidulid beetles are still a problem, suggesting that they can move large distances.

Michailides pointed that significant progress has been made in several areas on cultural practices and handling methods that affect aflatoxin contamination and presented some examples of some of the contributions in this area of research. An example will be the discovery of the source of contamination in Calimyrna and Conadria figs. Figs which drop on the surface of wet areas from drip irrigation are rehydrated and colonized by fungi including *A. flavus* (4%). This is a major source for replenishing the spore inoculum in the soil and can be a direct source of contamination after sweeping and mixing in these figs during harvesting. Isakeit suggested subsurface irrigation can be a good solution to avoid this kind of contamination. Michailides reported that the irrigation specialist Dr. Goldhamer has just started a project on subsurface irrigation.

Although Michailides jokingly mentioned that most of the molecular people had left before the end of the workshop, he was pleased that there were some of these researchers (G. Payne, D. Batnagar, N. Keller, and some others) still present. This was a good sign that agronomic practices, such as production and handling as approaches to reduce aflatoxin, had interested some of the researchers involved with molecular approaches. Understanding of the aflatoxin biosynthesis, developing resistant cultivars by either conventional breeding or molecular engineering, using biological control agents, developing "expert systems," improving sampling techniques, and agronomic practices, all are necessary for the most effective reduction of aflatoxin. Only with advances in all these areas of research will we be able to solve this complex puzzle.

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